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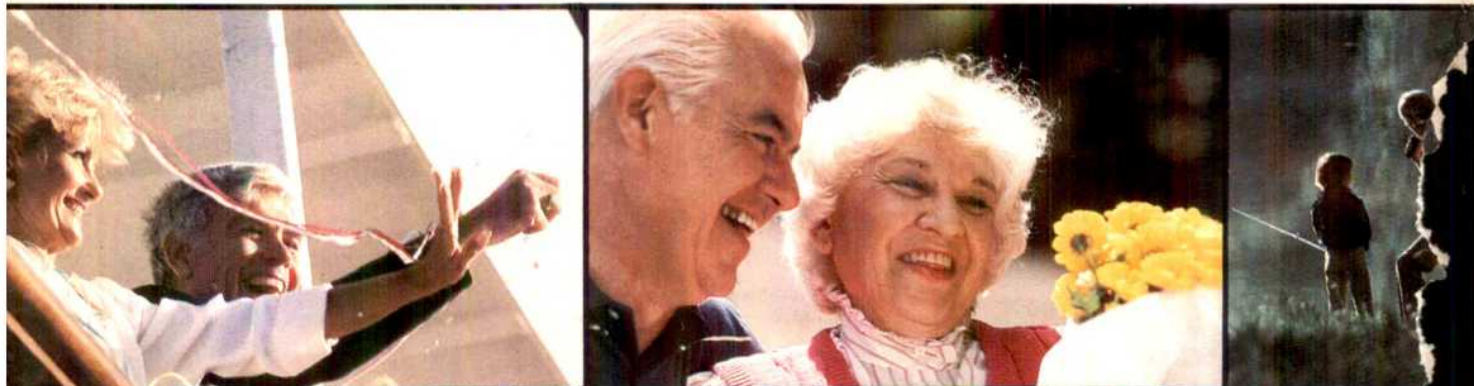
BLOOD

*The Journal of
The American Society of
Hematology*

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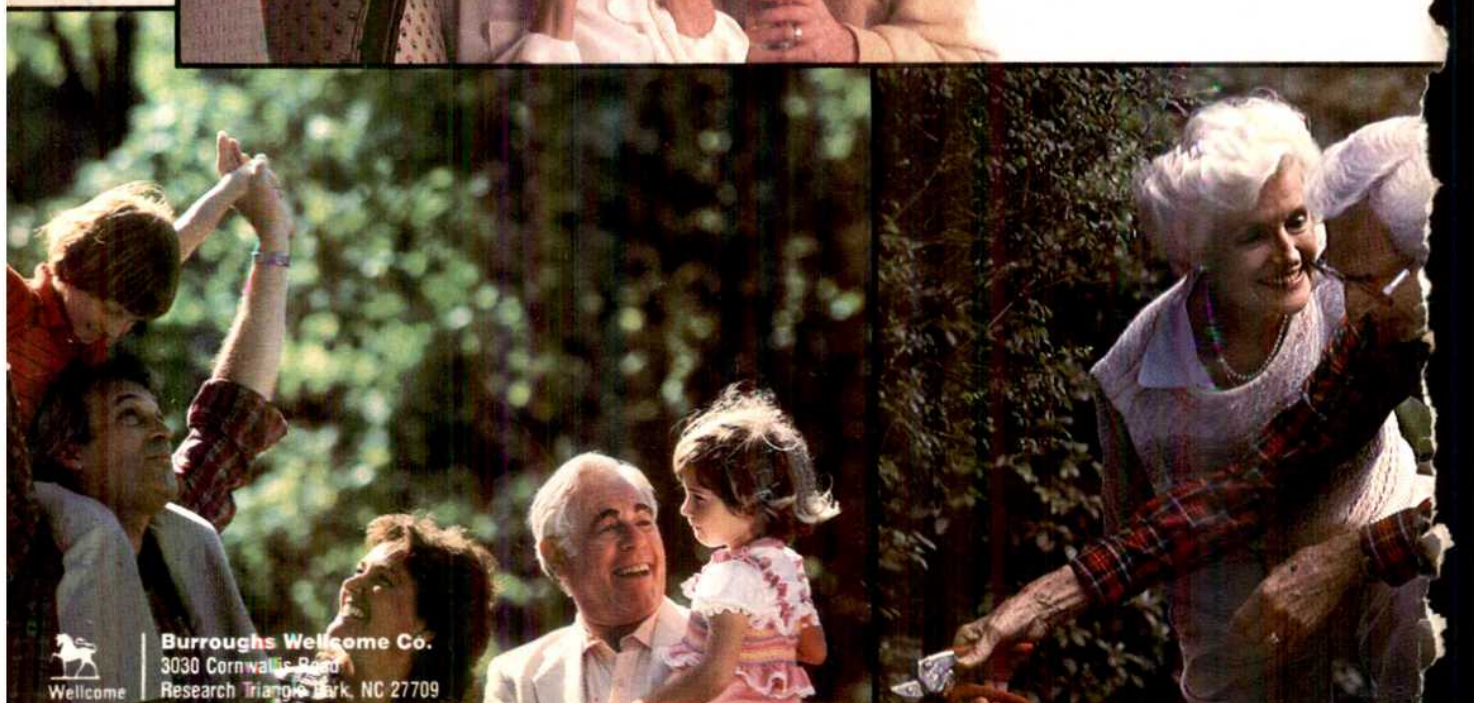
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For thousands of patients, Wellcovorin Tablets have provided the critical blood levels needed for life-saving rescue. The unique formulation of Wellcovorin Tablets has been shown to be completely bioavailable in a bioavailability study of 120 normal volunteers.¹

¹Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.



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Wellcovorin Tablets have reduced the cost of leucovorin rescue significantly. Before Wellcovorin Tablets, the cost of leucovorin increased rapidly and consistently every year. Now, patients pay less for Wellcovorin Tablets, and often realize considerable savings from a reduction in hospital stays and clinic visits.



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For greater flexibility in oral dosing, Wellcovorin Tablets are available in two sizes: 5 mg (scored, bottles of 20 or 100) and 25 mg (scored, bottles of 25).

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Leucovorin in convenient
5 mg and 25 mg tablets

Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B₁₂. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g., methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below $5 \times 10^{-8} M$.^{1,2} The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e., trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.^{3,4,5}

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Since men and women are equally affected by this disease, we urge everyone over 50 to get regular checkups.

The warning signs for colorectal cancer are a change in bowel habits and blood in the stool.

People with a family history of colon or rectal cancer or ulcerative colitis are at higher risk and are urged to be doubly cautious.

Checkup Guidelines for men and women over 50 without symptoms:

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- stool blood test annually
- procto exam every 3 to 5 years after 2 negative tests 1 year apart.

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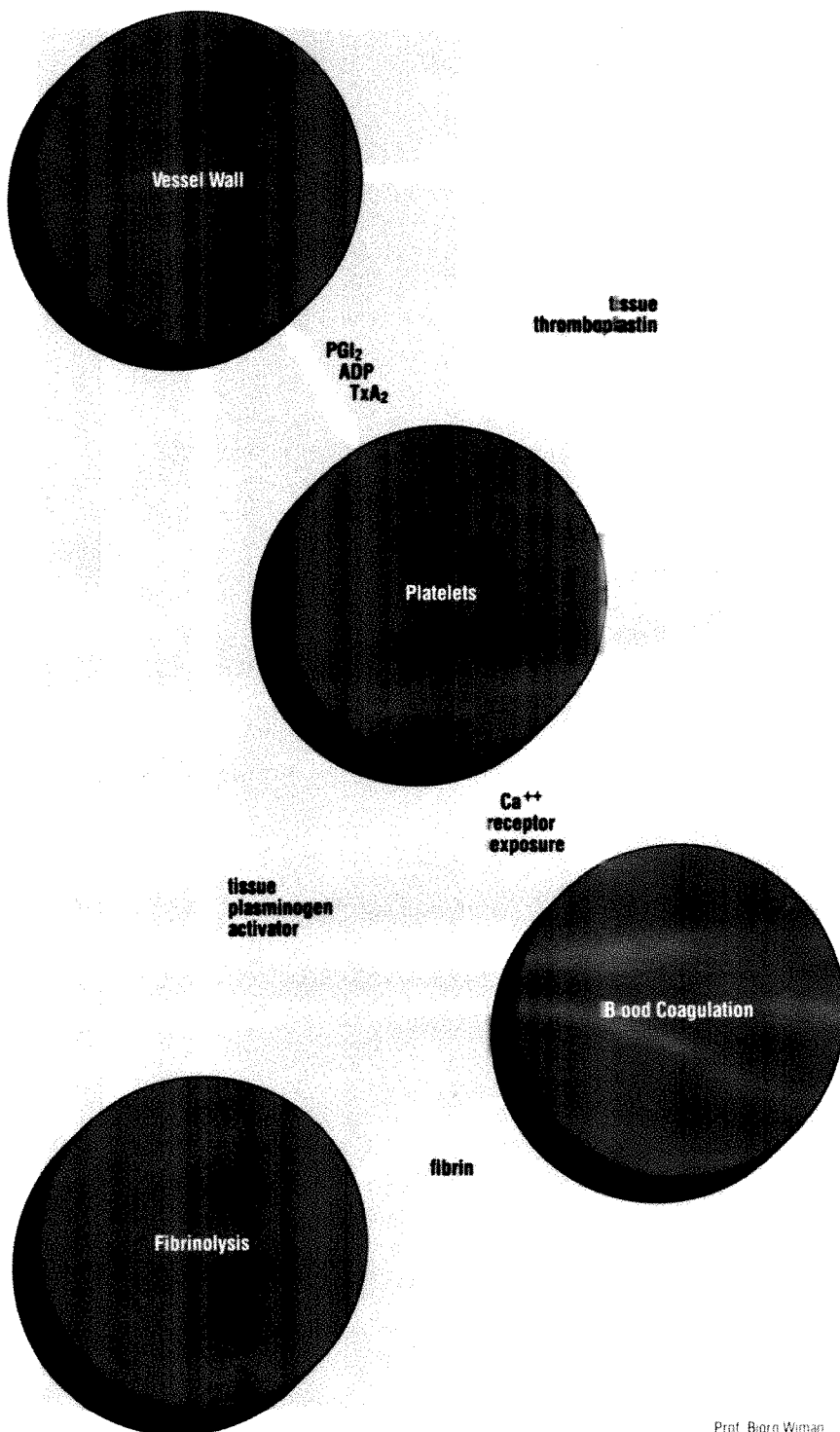
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See answer under flap. 

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Both slides are herpes simplex. You may be surprised, but especially in immunocompromised patients it is often difficult to differentiate between herpes infections and mucositis resulting from radiation therapy. Herpes simplex does not always have a classic presentation, and is easily misjudged.

Please see following page for brief summary of prescribing information.

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

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Herpes Simplex Infections in Immunocompromised Patients

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A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.⁴

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.⁵

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.⁶

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS AND DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuring renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M²) over an hour was associated with a lower frequency—4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used

in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the IV fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitteriness, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 AND HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M² at a constant rate over 1 hour, every 8 hours (750 mg/M²/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M ²)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

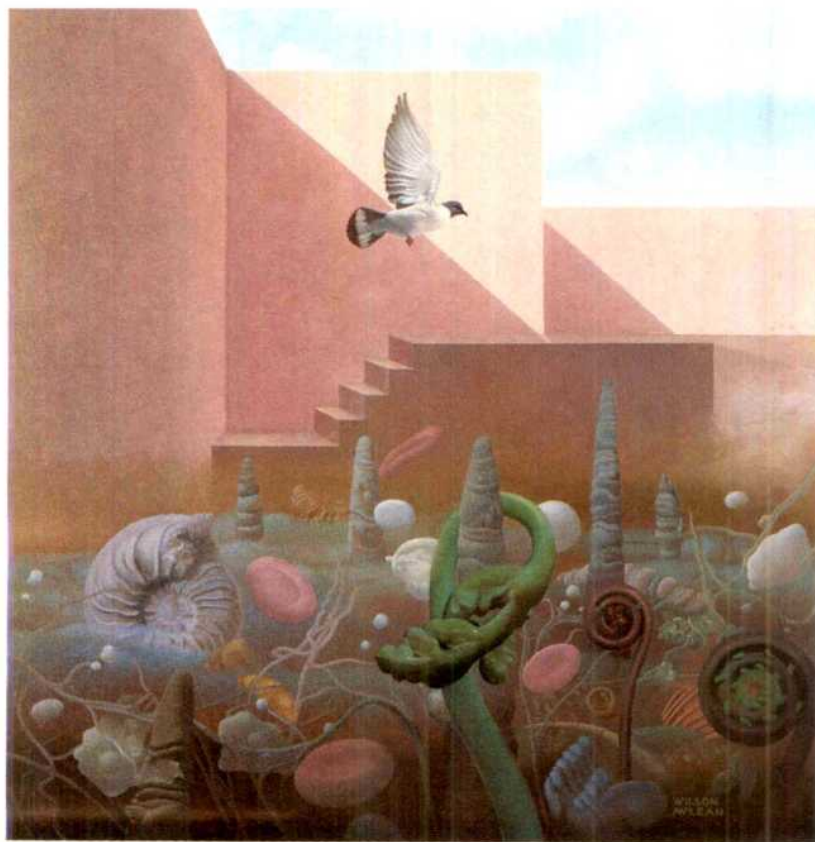
Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, *et al.*, *Lancet* (18235): 1389-1392, Jun. 27, 1986. 2. Wade, *et al.*, *Ann Intern Med.* 96(3): 265-269, Mar. 1982. 3. J.D. Meyers, *et al.*, *Am J Med* 73(1A): 229-235, Jul. 20, 1982. 4. Data on file, Burroughs Wellcome Co. 5. A. Mindel, *et al.*, *Lancet* (18274): 697-700, Mar. 27, 1982. 6. Z.M. Naib, *et al.*, *Cancer Res.* 33:1452-1463, 1973.

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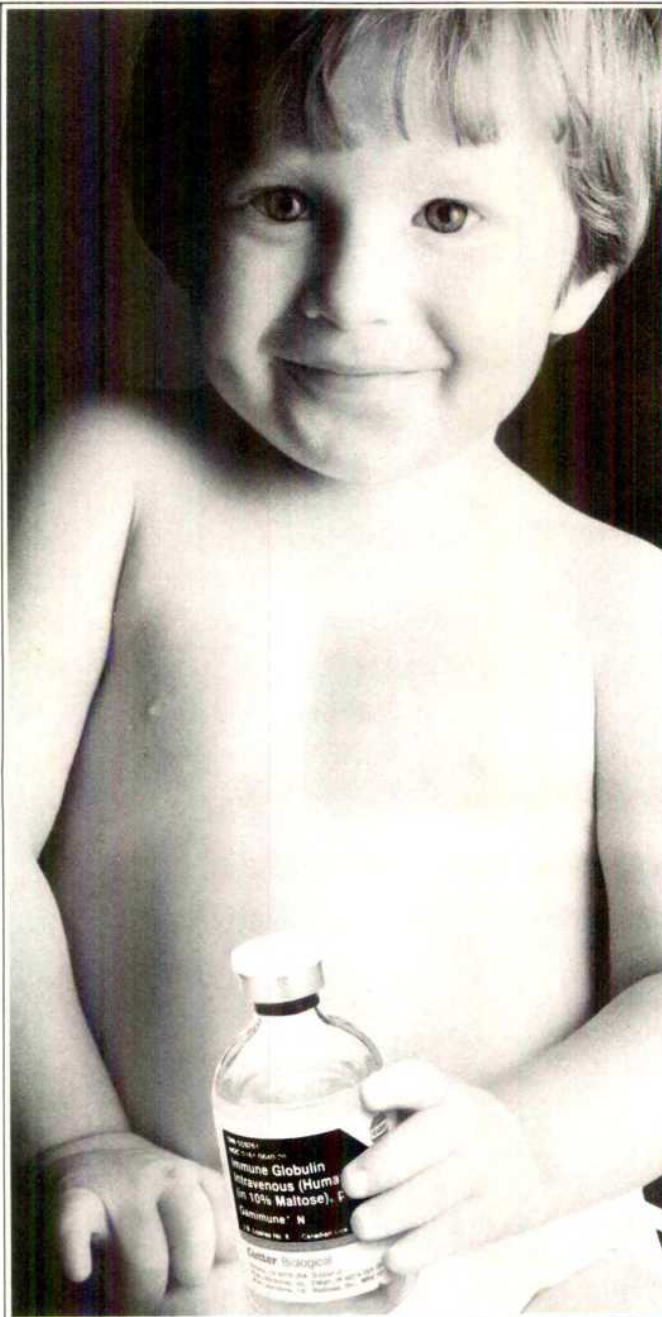
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(in 10% Maltose), pH 4.25



Documented *in vitro* inactivation of HTLV-III virus[‡]

[‡]The Cohn-Oncley process used to produce Gamimune® N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune® N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 TCID (Tissue Culture Infectious Doses) reduction of HTLV-III.[‡]

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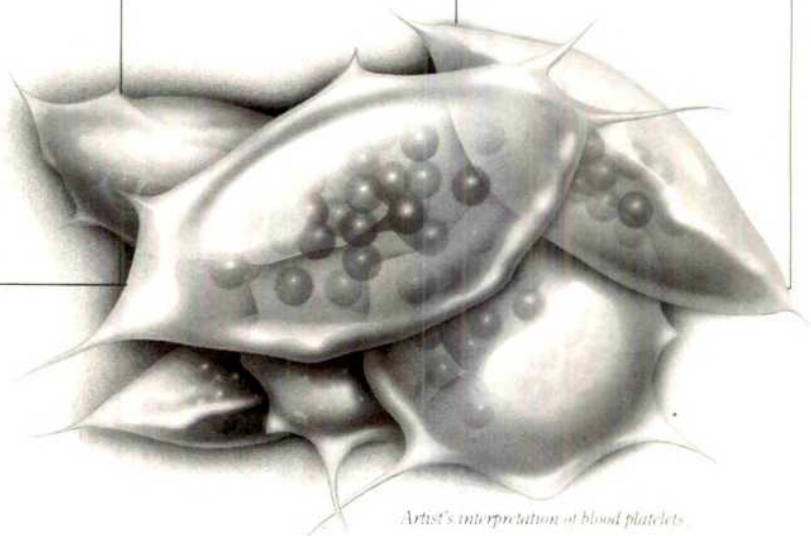
GAMIMUNE® N is manufactured without chemical modification. This means GAMIMUNE® N antibodies maintain normal opsonization capacity, have a normal distribution of IgG subgroups and provide the immunologic activity of normal plasma.[†]



Artist's interpretation of HTLV-III virus.

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Artist's interpretation of blood platelets.

*It is not possible to predict which patients with ITP will respond to therapy.

†In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days, transient (most often lasting from several days to several weeks) and should not be considered curative.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

GAMIMUNE[®] N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gammune[®] N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gammune[®] N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The *in vivo* half-life of Gammune[®] N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood, there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gammune[®] N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gammune[®] N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gammune[®] N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gammune[®] N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gammune[®] N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gammune[®] N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gammune[®] N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gammune[®] N may initiate a therapeutic increase in the platelet count. In clinical studies of Gammune[®] N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gammune[®] N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gammune[®] N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gammune[®] N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gammune[®] N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gammune[®] N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PLT⁺ alloimmune antibody with platelet anti PLT⁺ specificity) also responded to treatment with Gammune[®] N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gammune[®] N should be considered, in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days) transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gammune[®] N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gammune[®] N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gammune[®] N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gammune[®] N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gammune[®] N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gammune[®] N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gammune[®] N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gammune[®] N. It is not known whether Gammune[®] N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gammune[®] N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gammune[®] N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gammune[®] N. Symptoms reported with Gammune[®] N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gammune[®] N at the infusion site was reported in some cases.

In further studies of Gammune[®] N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gammune[®] N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gammune[®] N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gammune[®] N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gammune[®] N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gammune[®] N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gammune[®] N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gammune[®] N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gammune[®] N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gammune[®] N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes, if well tolerated, the rate may be gradually increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

U.S. License No. 8

REFERENCES: 1. GAMMUNE[®] N Product License Application. 2. Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(Jul-Aug): S382-S390. 3. Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26(4): 394-397.

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90% of Complete Responders

Disease-Free at Two Years*

MACOP-B

Drug Doses and Treatment Schedule for MACOP-B†

Drug	Dose	Route	Timing
Methotrexate††	400 mg/m ²	IV	Weeks 2, 6, 10
Doxorubicin	50 mg/m ²	IV	Weeks 1, 3, 5, 7, 9, 11
Cyclophosphamide	350 mg/m ²	IV	Weeks 1, 3, 5, 7, 9, 11
Vincristine	1.4 mg/m ²	IV	Weeks 2, 4, 6, 8, 10, 12
Bleomycin	10 U/m ²	IV	Weeks 4, 8, 12
Prednisone	75 mg	Oral	Daily, dose tapered over the last 15 days
Co-trimoxazole	2 tablets	Oral	Twice daily throughout

† MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin.
IV = intravenous.

†† Methotrexate given as a 100 mg/m² intravenous bolus, then 300 mg/m² intravenously over 4 hours, followed 24 hours later by folic acid, 15 mg orally, every 6 hours for 6 doses.

* Himm P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med*. 1985;102:596-602, 1985.

Blenoxane®

(sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin; larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and

dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces non-specific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes do not predict the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are non-specific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO}

falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

- (1) Maintain FiO₂ at concentrations approximately that of room air (25%) during surgery and the post-operative period.
- (2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

SUPPLY: Each vial contains 15 units of Blenoxane as sterile bleomycin sulfate. NDC 0015-3010-20.

UA-270

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PREPARING THE MANUSCRIPT

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The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which work was done; (4) acknowledgments for research support; (5) name and address of the author to whom communications regarding the manuscript should be directed.

The second page should contain an abstract of 200 words or less, summarizing the reason for the study, the methods used, the results, and the major conclusions. Do not include a summary at the end of the paper.

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Figures, especially charts, graphs, and line drawings, are generally reduced in size for publication (consult a recent issue of the journal for examples). To maintain legibility it is important that all numbers, letters, and symbols be large enough originally so that when reduced they will remain at least ⅛ inch (2 mm) high (ie, approximately the same size as used for this line of type). Figures not properly prepared will have to be returned to the contributor for revision or will be relettered by the printer and the cost charged to the contributor.

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Failure to comply with the specifications for preparing illustrations and tables may result in a delay of the review process.

REFERENCES

References should be compiled at the end of the article according to the order of citation in the text. They should be typewritten, double spaced, under the heading REFERENCES. Abbreviations for titles of medical periodicals should conform to those used in the latest edition of *Index Medicus*. (A "List of Journals Indexed in Index Medicus"—with abbreviations—is obtainable from the Superintendent of Documents, US Government Printing Office, Washington, DC 20402, at a modest charge.) Name all authors. Supply only first page number for each reference. References to abstracts and letters must be identified as such.

Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

EXAMPLES OF REFERENCES

Journal article, one or more authors:

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PROOFREADING

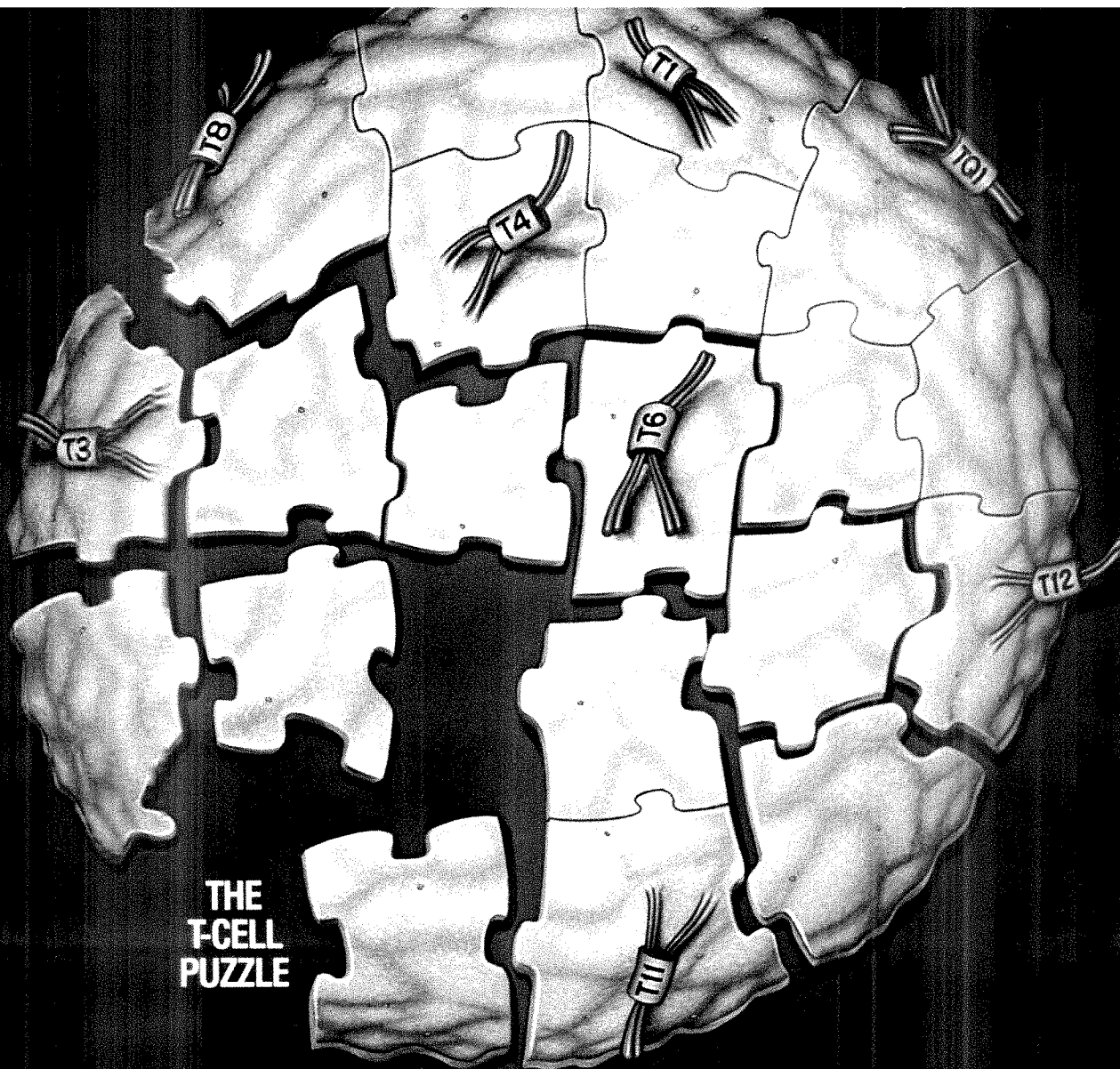
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a breakthrough in hairy cell leukemia

- Effective in all stages of the disease.
- Statistically increased survival compared to a historical control group.
- Normalizes peripheral blood elements.
- Improves quality of life.
- Dramatically decreases the need for red blood cell and platelet transfusions.
- Virtually eliminates life-threatening opportunistic infections after several months of therapy.
- Ready-to-use injectable solution—no mixing or reconstitution.
- Also available in sterile powder for injection with diluent for reconstitution.
- SC or IM injection and fixed dosing permit administration without weight or body surface area calculation in home, office or institution.
- Caution patients not to change brands of Interferon without medical consultation, as a change in dosage may result.
- Common initial side effects, such as fever, chills, myalgia, headache and fatigue, are self-limiting and generally manageable. Myelosuppression, which can occur early in the treatment course, warrants close clinical and laboratory observation and is usually alleviated by dose reduction or temporary discontinuation of drug. Persistent fatigue is found to be less troublesome with *p.m.* or *h.s.* administration.



Injectable Solution—
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18 million IU vial (3 million IU/0.5 mL)

Sterile Powder for Injection—
18 million IU and 3 mL diluent for reconstitution (3 million IU/0.5 mL)

NEW

SC/IM

ROFERON[®]-A

BRAND OF
Interferon

alfa-2a, recombinant/Roche

a promise fulfilled

Before prescribing, please see complete product information on following pages.

NEW Roferon-A[®] SC/IM Interferon alfa-2a, recombinant/Roche

DESCRIPTION: Roferon-A (Interferon alfa-2a, recombinant, Roche) is a sterile protein product for use by injection. Roferon-A is manufactured by recombinant DNA technology that employs a genetically engineered *E. coli* bacterium containing DNA that codes for the human protein, Interferon alfa-2a, recombinant. Roche is a highly purified protein containing 165 amino acids, and it has an approximate molecular weight of 19,000 daltons. The purification procedure includes affinity chromatography using a murine monoclonal antibody. Fermentation is carried out in a defined nutrient medium containing the antibiotic tetracycline hydrochloride, 5 mg/liter. However, the presence of the antibiotic is not detectable in the final product. Roferon-A is supplied as an injectable solution or as a sterile powder for injection with its accompanying diluent.

Injectable Solution: The solution is colorless and contains Interferon alfa-2a, recombinant, Roche, sodium chloride, Albumin (Human) and phenol as a preservative.

Sterile Powder for Injection: The powder is white to beige and contains Interferon alfa-2a, recombinant, Roche, sodium chloride and Albumin (Human).

Diluent for Sterile Powder for Injection: The diluent contains sodium chloride, Albumin (Human) and phenol as a preservative. Each vial of Roferon-A contains 3 million or 18 million international units (IU) of Interferon alfa-2a, recombinant, Roche. The specific activity of Interferon alfa-2a, recombinant, Roche is 2×10^6 IU/mg protein. The route of administration is subcutaneous or intramuscular.

CLINICAL PHARMACOLOGY: The mechanism by which Interferon alfa-2a, recombinant, Roche, or any other interferon, exerts antitumor activity is not clearly understood. However, it is believed that direct antiproliferative action against tumor cells and modulation of the host immune response play important roles in the antitumor activity.

The biological activities of Interferon alfa-2a, recombinant, Roche are species-restricted, i.e., they are expressed in a very limited number of species other than humans. As a consequence, preclinical evaluation of Interferon alfa-2a, recombinant, Roche has involved *in vitro* experiments with human cells and some *in vivo* experiments. Using human cells in culture, Interferon alfa-2a, recombinant, Roche has been shown to have antiproliferative and immunomodulatory activities that are very similar to those of the mixture of interferon alfa subtypes produced by human leukocytes. *In vivo*, Interferon alfa-2a, recombinant, Roche has been shown to inhibit the growth of several human tumors growing in immunocompromised (nude) mice. Because of its species-restricted activity, it has not been possible to demonstrate antitumor activity in immunologically intact syngeneic tumor model systems, where effects on the host immune system would be observable. However, such antitumor activity has been repeatedly demonstrated with, for example, mouse interferon-alfa in transplantable mouse tumor systems. The clinical significance of these findings is unknown. The metabolism of Interferon alfa-2a, recombinant, Roche is consistent with that of alpha interferons in general. Alpha interferons are totally filtered through the glomeruli and undergo rapid proteolytic degradation during tubular reabsorption, rendering a negligible reappearance of intact alpha interferon in the systemic circulation. Small amounts of radiolabeled Interferon alfa-2a, recombinant, Roche appear in the urine of isolated rat kidneys, suggesting near complete reabsorption of Interferon alfa-2a, recombinant, Roche catabolites. Liver metabolism and subsequent biliary excretion are considered minor pathways of elimination for alpha interferons.

The serum concentrations of Interferon alfa-2a, recombinant, Roche reflected a large intersubject variation in both healthy volunteers and patients with disseminated cancer.

In healthy people, Interferon alfa-2a, recombinant, Roche exhibited an elimination half-life of 3.7 ± 8.5 hours (mean 5.1 hours), volume of distribution at steady state of 0.223 ± 0.748 L/kg (mean 0.400 L/kg) and a total body clearance of 2.14 to 3.62 mL/min/kg (mean 2.79 mL/min/kg) after a 36 million IU (2.2×10^6 pg) intravenous infusion. After intramuscular and subcutaneous administrations of 36 million IU, peak serum concentrations ranged from 1500 to 2580 pg/mL (mean 2020 pg/mL) at a mean time to peak of 3.8 hours and from 1250 to 2320 pg/mL (mean 1730 pg/mL) at a mean time to peak of 7.3 hours, respectively. The apparent fraction of the dose absorbed after intramuscular injection was greater than 80% .

The pharmacokinetics of Interferon alfa-2a, recombinant, Roche after single intramuscular doses to patients with disseminated cancer were similar to those found in healthy volunteers. Dose proportional increases in serum concentrations were observed after single doses up to 198 million IU. There were no changes in the distribution or elimination of Interferon alfa-2a, recombinant, Roche during twice daily (0 to 36 million IU), once daily (1 to 54 million IU), or three times weekly (1 to 135 million IU) dosing regimens up to 28 days of dosing. Multiple intramuscular doses of Interferon alfa-2a, recombinant, Roche resulted in an accumulation of 2 to 4 times the single dose serum concentrations. Pharmacokinetic information in patients with hairy cell leukemia is presently unknown.

The acute parenteral toxicity of Interferon alfa-2a, recombinant, Roche has been studied in mice, rats, rabbits and ferrets at doses up to 30 million IU/kg intravenously, and 500 million IU/kg intramuscularly. No treatment-related mortality was noted in any species given Interferon alfa-2a, recombinant, Roche by any of the routes of administration.

Effects on Hairy Cell Leukemia: During the first one to two months of treatment of patients with hairy cell leukemia, significant depression of hematopoiesis was likely to occur. Subsequently, there was improvement in circulating blood cell counts.

Of the 75 patients who were evaluable for efficacy following at least 16 weeks of therapy, 46 (61%) achieved complete or partial response. Twenty-one patients (28%) had a minor remission, eight (11%) remained stable, and none had worsening of disease. All patients who achieved either a complete or partial response had complete or partial normalization of all peripheral blood elements including hemoglobin level, white blood cell, neutrophil, monocyte and platelet counts with a concomitant decrease in peripheral blood and bone marrow hairy cells. Responding patients also exhibited a marked reduction in red blood cell and platelet transfusion requirements, a decrease in infectious episodes and improvement in performance status. The probability of survival for two years in patients receiving Roferon-A (94%) was statistically increased compared to a historical control group (75%).

ROFERON-A (Interferon alfa-2a, recombinant/Roche)

INDICATIONS AND USAGE: Roferon-A is indicated for use in the treatment of hairy cell leukemia in people 18 years of age or older. Studies have shown that Roferon-A can produce clinically meaningful regression or stabilization of this disease, both in previously splenectomized and nonsplenectomized patients.^{2,3} Prior to initiation of therapy, tests should be performed to quantitate peripheral blood hemoglobin, platelets, granulocytes and hairy cells and bone marrow hairy cells. These parameters should be monitored periodically (e.g., monthly) during treatment to determine whether response to treatment has occurred. If a patient does not respond within six months, treatment should be discontinued. If a response to treatment does occur, treatment should be continued until no further improvement is observed and these laboratory parameters have been stable for about three months. It is not known whether continued treatment after that time is beneficial. Studies are in progress to evaluate this question.

CONTRAINDICATIONS: Roferon-A is contraindicated in patients with known hypersensitivity to alpha interferon, mouse immunoglobulin or any component of the product.

WARNINGS: Roferon-A should be administered under the guidance of a qualified physician. (See DOSAGE AND ADMINISTRATION.) Appropriate management of the therapy and its complications is possible only when adequate diagnostic and treatment facilities are readily available.

Roferon-A should be used with caution in patients with severe preexisting cardiac disease, severe renal or hepatic disease, seizure disorders and/or compromised central nervous system function.

Because of the possibility of severe or even fatal adverse reactions, patients should be informed not only of the benefits of therapy but also of the risks involved.

Roferon-A should be administered with caution to patients with cardiac disease or with any history of cardiac illness. No direct cardiotoxic effect has been demonstrated, but it is likely that acute, self-limited toxicities (i.e., fever, chills) frequently associated with Roferon-A administration may exacerbate preexisting cardiac conditions. Rarely, myocardial infarction has occurred in patients receiving Roferon-A.

Caution should be exercised when administering Roferon-A to patients with myelosuppression.

Central nervous system adverse reactions have been reported in a number of patients. These reactions included decreased mental status, exaggerated central nervous system function, and dizziness. More severe obtundation and coma have been rarely observed. Most of these abnormalities were mild and reversible within a few days to three weeks upon dose reduction or discontinuation of Roferon-A therapy. Careful periodic neuropsychiatric monitoring of all patients is recommended.

Leukopenia and elevation of hepatic enzymes occurred frequently but were rarely dose-limiting. Thrombocytopenia occurred less frequently. Proteinuria and increased cells in urinary sediment were also seen infrequently. Rarely, significant hepatic, renal and myelosuppressive toxicities were noted.

PRECAUTIONS: *General:* In all instances where the use of Roferon-A is considered for chemotherapy, the physician must evaluate the need and usefulness of the drug against the risk of adverse reactions. Most adverse reactions are reversible if detected early. If severe reactions occur, the drug should be reduced in dosage or discontinued and appropriate corrective measures should be taken according to the clinical judgment of the physician. Reinstitution of Roferon-A therapy should be carried out with caution and with adequate consideration of the further need for the drug and alertness as to possible recurrence of toxicity. The minimum effective dose of Roferon-A for treatment of hairy cell leukemia has not been established.

Information for Patient: Patients should be cautioned not to change brands of interferon without medical consultation, as a change in dosage may result. Patients should be informed regarding the potential benefits and risks attendant to the use of Roferon-A. If home use is determined to be desirable by the physician, instructions on appropriate use should be given, including review of the contents of the enclosed Patient Information Sheet. Patients should be well hydrated, especially during the initial stages of treatment.

Laboratory Tests: Periodic complete blood counts and liver function tests should be performed during the course of Roferon-A treatment. They should be performed prior to therapy and at appropriate periods during therapy. Since responses of hairy cell leukemia are not generally observed for one to three months after initiation of treatment, very careful monitoring for severe depression of blood cell counts is warranted during the initial phase of treatment. Those patients who have preexisting cardiac abnormalities and/or are in advanced stages of cancer should have electrocardiograms taken prior to and during the course of treatment.

Carcinogenesis, Mutagenesis and Impairment of Fertility:

Carcinogenesis: Roferon-A has not been tested for its carcinogenic potential. *Mutagenesis:* A. Internal studies—Ames tests using six different tester strains, with and without metabolic activation, were performed with Roferon-A up to a concentration of 1920 µg/plate. There was no evidence of mutagenicity. Human lymphocyte cultures were treated *in vitro* with Roferon-A at noncytotoxic concentrations. No increase in the incidence of chromosomal damage was noted. B. Published studies—There are no published studies on the mutagenic potential of Roferon-A. However, a number of studies on the genotoxicity of human leukocyte interferon have been reported.

A chromosomal defect following the addition of human leukocyte interferon to lymphocyte cultures from a patient suffering from a lymphoproliferative disorder has been reported.

In contrast, other studies have failed to detect chromosomal abnormalities following treatment of lymphocyte cultures from healthy volunteers with human leukocyte interferon.

It has also been shown that human leukocyte interferon protects primary chick embryo fibroblasts from chromosomal aberrations produced by gamma rays. *Impairment of Fertility:* Roferon-A has been studied for its effect on fertility in Macaca mulatta (rhesus monkeys). Nonpregnant rhesus females treated with Roferon-A at doses of 5 and 25 million IU/kg day have shown menstrual cycle irregularities, including prolonged or shortened menstrual periods and erratic bleeding; these cycles were considered to be anovulatory on the basis that reduced progesterone levels were noted and that expected increases in preovulatory estrogen and luteinizing hormones were not observed. These monkeys returned to a normal menstrual rhythm following discontinuation of treatment. *Drug Interactions:* Interactions between Roferon-A and other drugs have not been fully evaluated.

PREGNANCY: Teratogenic Effects: Pregnancy Category C. Roferon-A has been shown to demonstrate a statistically significant increase in abortifacient activity in rhesus monkeys when given at approximately 20 to 500 times the human dose. A study in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg day of Roferon-A in their early to midfetal period (days 22 to 70 of gestation) has failed to demonstrate teratogenic activity for Roferon-A. There are no adequate and well-controlled studies in pregnant women. *Nonteratogenic Effects:* Dose-related abortifacient activity was observed in pregnant rhesus monkeys treated with 1, 5 or 25 million IU/kg day of Roferon-A.

ROFERON[®]-A (Interferon alfa-2a, recombinant Roche)

in their early to mid fetal period (days 22 to 70 of gestation). A late-fetal period (days 79 to 100 of gestation) is in progress and as yet there have been no reports of any increase in rate of abortion.

Usage in Pregnancy. Safe use in human pregnancy has not been established. Therefore, Roferon-A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Information from primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Increases in serum estradiol and progesterone concentrations have been reported in women treated with human leukocyte interferon- α . Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during the therapy period.

Male fertility and teratogenic evaluations have yielded no significant adverse effects to date.

Nursing Mothers. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Roferon-A, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use. Safety and effectiveness in children under 18 years of age have not been established.

ADVERSE REACTIONS. The following data on adverse reactions are based on the subcutaneous or intramuscular administration of Roferon-A as a single agent for the treatment of 53 patients with hairy cell leukemia during investigational trials in the United States.

Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%) and headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (2%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), sinusitis or inflammation of the oropharynx (16%), dry skin or pruritus (13%), weight loss (14%) were observed with moderate frequency. Less commonly, dyspareunia (8%), paresthesias (6%), numbness (6%), partial alopecia (5%), reactivation of herpes labialis (8%), transient impotence (6%) and arthralgia (5%) were also observed. Rarely (< 3%), central nervous system effects including decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias and palpitations, were reported. Adverse experiences which occurred rarely and may have been related to underlying disease included epistaxis, bleeding gums, ecchymosis and petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis and inflammation at the site of injection, were also rarely observed. Roferon-A has a safety profile similar to that of the treatment of many other types of cancer under investigational trials in the United States. These studies generally utilized higher doses (10 to 50 million IU/m²). All of the previously described adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in patients who received higher doses of Roferon-A. Some additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%) and edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: **Gastrointestinal**—abdominal fullness, hypermotility and heartburn; **Central Nervous System**—gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dyspraxia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia and loss of libido; **Peripheral Nervous System**—muscle contractions; **Cardiovascular**—congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon and hot flashes; **Pulmonary**—bronchospasm and tachypnea; **Miscellaneous**—excessive salivation and flushing of skin. **Abnormal Laboratory Test Values.** The percentage of patients with hairy cell leukemia or with other types of malignancies who experienced a significant abnormal laboratory test value at least once during their treatment is shown in the following table.

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary* (n = 1019)
Hematologic		
Leukopenia	59%	69%
Neutropenia	39%	58%
Thrombocytopenia	42%	42%
Decreased Hemoglobin	36%	6.3%
Decreased Hematocrit	43%	12.5%
Mean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
Hepatic		
SGOT	47%	78%
Alkaline Phosphatase	18%	48%
LDH	12%	47%
Bilirubin	1.6%	31%
Renal/Urinary		
BUN	2%	10%
Serum Creatinine	3%	10%
Uric Acid†	6%	15%
Proteinuria‡	10%	25%
Other Chemistry Tests		
Hypocalcemia	10%	51%
Elevated Serum Glucose§	33%	39%
Elevated Serum Phosphorus	2%	17%

*Patients with hairy cell leukemia are included in this overall summary.

†The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.

‡Random glucose determination.

§Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic

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lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count < 50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended. Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.

The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count < 50,000) or who are at risk for bleeding.

Injectable Solution:

3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant Roche, 9 mg sodium chloride, 5 mg Albumin (Human) and 3 mg phenol as a preservative.

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Sterile Powder for Injection:

18 million IU Roferon-A per vial—Reconstitute with 3 mL diluent and swirl gently to dissolve. When reconstituted with Diluent for Roferon-A, each 1 mL of reconstituted solution contains 6 million IU Interferon alfa-2a, recombinant Roche, 9 mg sodium chloride, 5 mg Albumin (Human) and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant Roche. Once the powder is reconstituted, it must be used within thirty (30) days.

The sterile powder and its accompanying diluent, the reconstituted solution and the injectable solution should be stored in the refrigerator at 36° to 45° F (2° to 8° C). Do not freeze or shake.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

HOW SUPPLIED:

Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01).

Roferon-A Injectable Solution: Vials containing 18 million IU Interferon alfa-2a, recombinant Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09).

Roferon-A Sterile Powder for Injection: Vials containing 18 million IU Interferon alfa-2a, recombinant Roche, with accompanying diluent. Boxes of 1 (NDC 0004-1993-09).

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†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

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Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 36°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. 2. Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. 3. Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 399:1201-1203, 1978. 4. Data on file, Miles Pharmaceuticals.



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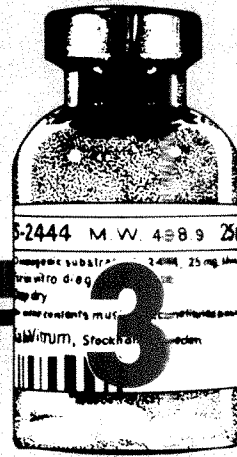
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BRIEF REVIEW

Subunit Assembly of Hemoglobin: An Important Determinant of Hematologic Phenotype

By H. Franklin Bunn

Hemoglobin's physiologic properties depend on the orderly assembly of its subunits in erythropoietic cells. The biosynthesis of α - and β -globin polypeptide chains is normally balanced. Heme rapidly binds to the globin subunit, either during translation or shortly thereafter. The formation of the $\alpha\beta$ -dimer is facilitated by electrostatic attraction of a positively charged α -subunit to a negatively charged β -subunit. The $\alpha\beta$ -dimer dissociates extremely slowly. The difference between the rate of dissociation of $\alpha\beta$ - and $\alpha\gamma$ -dimers with increasing pH explains the well-known alkaline resistance of Hb F. Two dimers combine to form the functional $\alpha_2\beta_2$ -tetramer. This model of hemoglobin assembly explains the different levels of positively charged and negatively charged mutant hemoglobins that are encountered in heterozygotes and the effect of α -thalassaemia and heme deficiency states in modifying the level of the

variant hemoglobin as well as Hb A₂. Electrostatic interactions also affect the binding of hemoglobin to the cytoplasmic surface of the red cell membrane and may underlie the formation of target cells. Enhanced binding of positively charged variants such as S and C trigger a normally dormant pathway for potassium and water loss. Thus, the positive charge on β^c is responsible for the two major contributors to the pathogenesis of Hb SC disease: increased proportion of Hb S and increased intracellular hemoglobin concentration. It is likely that electrostatic interactions play an important role in the assembly of a number of other multisubunit macromolecules, including membrane receptors, cytoskeletal proteins, and DNA binding proteins.

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JUST as the accuracy and durability of a fine watch depend on the well-engineered fit of its moving parts, many regulatory events in biology require dynamic interaction between the "moving parts" of multisubunit proteins. Hemoglobin is probably the most thoroughly studied example; its physiologic function depends on gear- and ratchet-like movement between its subunits.¹⁻³ Our understanding of how hemoglobin works at the molecular and atomic levels is based on contributions from many disciplines, including structural and physical biochemistry, molecular genetics, and clinical medicine. Lessons learned from hemoglobin can be applied with profit to a number of other multisubunit proteins, some of which are of special interest to hematologists.

A rigorous set of constraints is involved in the orderly construction of a multisubunit protein. In most instances, including hemoglobin, the unlike subunits are products of different genes. (Exceptions include insulin and haptoglobin, whose subunits are cleaved from a precursor polypeptide and linked by disulfide bonds.) Efficient biosynthesis of hemoglobin requires balanced production of α and β polypeptide chains. Moreover, enough heme must be synthesized in the mitochondria to provide each of the newly made globin chains with its all-important prosthetic group. The reaction between heme and apoglobin is rapid.^{4,5} Therefore, it is likely that heme is inserted into its proper niche either during translation of the nascent globin chain or shortly thereafter. A slight mismatch in globin chain biosynthesis can be tolerated because erythroid precursor cells have an efficient

adenosine triphosphate-dependent proteolytic system⁶⁻⁸ that attacks free subunits^{9,10} but leaves assembled dimers and tetramers unscathed. Likewise, excess heme can be catabolized by an inducible microsomal oxygenase.¹¹ Despite intensive experimental scrutiny, the mechanisms responsible for the precise coordination between the synthesis of globin subunits and that of heme are unclear. These good housekeeping practices are essential to normal red cell viability. The thalassemias are prototypes of chain imbalance. Most of the phenotypic features of both the β -thalassemias and the α -thalassemias can be explained by either the deleterious effect of free subunits on the red cell membrane or the accumulation of these relatively insoluble and noncooperative subunits in the cytosol.

SUBUNIT ASSEMBLY OF HEMOGLOBIN

Once equivalent amounts of α - and β -subunits have been translated on their respective polyribosomes, and once heme has been inserted, the hemoglobin subunits are now faced

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with the challenging task of diffusing within the thick tomato soup and finding unlike partner subunits so that they can assemble into the functional $\alpha_2\beta_2$ -tetramer. A growing body of hematologic and experimental evidence indicates that subtle alterations in the rate of subunit assembly are prime determinants of the distribution of hemoglobins seen in normal and pathologic red cells and can explain a number of hitherto puzzling phenotypic features.

The steps in hemoglobin assembly are depicted schematically in Fig 1. Note that a heme-intact β -subunit will combine with a heme-intact α -subunit or, alternatively, may aggregate with like β -subunits to form β_4 -tetramers or Hb H. Under normal circumstances the concentration of free β -subunits is likely to be sufficiently low that there is no significant amount of β_4 in the cytosol of erythropoietic cells. In contrast, in certain types of α -thalassemia, Hb H and its fetal counterpart Hb Barts (γ_4) can be detected, and in the case of hydrops fetalis (deletion of all four α -globin genes [---/---]), these homotetramers are virtually the only hemoglobins detected.

The critical and rate-limiting step in hemoglobin assembly is the simple bimolecular reaction $\alpha + \beta \rightarrow \alpha\beta$. This noncovalent reaction is nearly irreversible. As discussed later, the reverse reaction does occur but slowly. In contrast, the combination of two like $\alpha\beta$ -dimers to form the $\alpha_2\beta_2$ -tetramer is readily reversible.^{12,13} The interface at which these two dimers slide on each other is crucial to the cooperative behavior of hemoglobin.¹⁻³

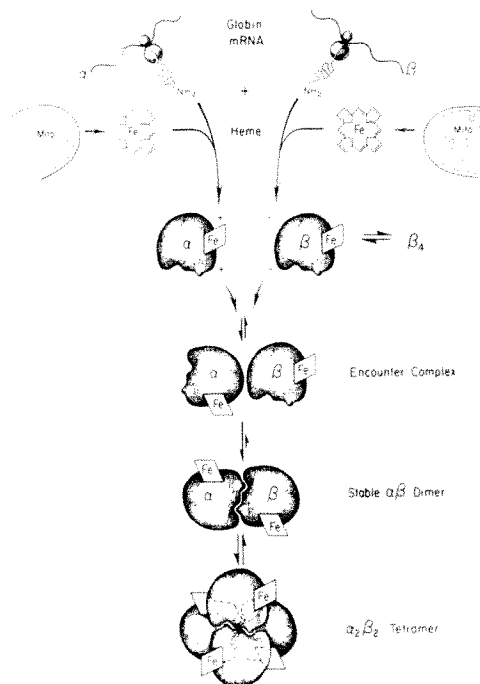


Fig 1. Assembly of hemoglobin. The α - and β -globin polypeptides are translated on their respective mRNAs. On binding of heme, the protein folds into its native three-dimensional structure. The binding of α - and β -hemoglobin subunits to each other is facilitated by electrostatic attraction. An unstable intermediate encounter complex can rearrange to form the stable $\alpha\beta$ -dimer. Two dimers combine to form the functional $\alpha_2\beta_2$ -tetramer.

SURVEY OF MUTANT HEMOGLOBINS

The vast body of information on the human hemoglobin variants can provide special insights into the mechanism of subunit assembly. Several independent factors influence the formation of normal and mutant hemoglobins.^{3,14} A few variant subunits are synthesized at a significantly lower rate than that of their normal counterparts. Examples include Hb E¹⁵⁻¹⁷ and Hb Knossos,¹⁸ in which the mutations lead to defective β -globin mRNA processing, and therefore significantly reduced protein synthesis and a thalassemic phenotype. In contrast, the vast majority of variant subunits appear to have normal rates of transcription and translation. Another factor that influences the amount of variant hemoglobin in the red cell is the stability of the variant subunit. Among the 70-odd unstable β -globin variants that have been reported to date, most constitute less than 30% of the total hemoglobin, owing to increased catabolism of the variant both in erythroid precursor cells and in the circulation.^{3,19}

A third factor, differences in the rates of subunit assembly, could explain the variability in the levels of most of the stable hemoglobin variants. Even small differences in the rates of combination of variant subunits with normal partner subunits could have a significant effect on the distribution of hemoglobins in the red cell. In order to explore this hypothesis, we surveyed reports in the literature on β -globin variants.²⁰ Humans inherit two β -globin genes, one from each parent. Accordingly, if a variant β -subunit is translated at a normal rate and has normal stability and solubility, red cells of heterozygotes would be expected to contain equal amounts of the normal and variant hemoglobin.* However, the reported values show unexpected variability. Many of the commonly encountered β -globin variants are present in significantly lower amounts than in Hb A. As shown in Fig 2, these variants tend to be positively charged. In the presence of α -thalassemia, where there is a limiting amount of α -chains, the proportion of the positively charged variant is further decreased in proportion to the number of α -globin genes deleted. In contrast, negatively charged variants are often present in heterozygote red cells in amounts greater than Hb A. In one case (Hb J-Baltimore), the presence of α -thalassemia resulted in an increase in the proportion of the variant. Another variant (Hb N-Baltimore) was unchanged by the presence of α -thalassemia.

The levels of the stable β -globin variants shown in Fig 2 are independent of the location of the amino acid substitution on the surface of the molecule. All substitutions at the interfaces between α and β subunits were excluded from this analysis because structural alterations at these sites could have a direct effect on hemoglobin assembly independent of charge. Moreover, α -globin variants were excluded for two reasons. First, the levels of α -globin mutants are markedly affected by α -thalassemia, a condition that is often not well

*In normal red cells, the minor components, Hb A₂, F, and the glycosylated hemoglobins (Hb A_{1c}, A_{1b}, etc) compose about 10% of the total hemoglobin. Therefore, in β -globin heterozygotes the variant and normal Hb A would each constitute 45% of the total if they had equal rates of synthesis and assembly as well as equal stability.

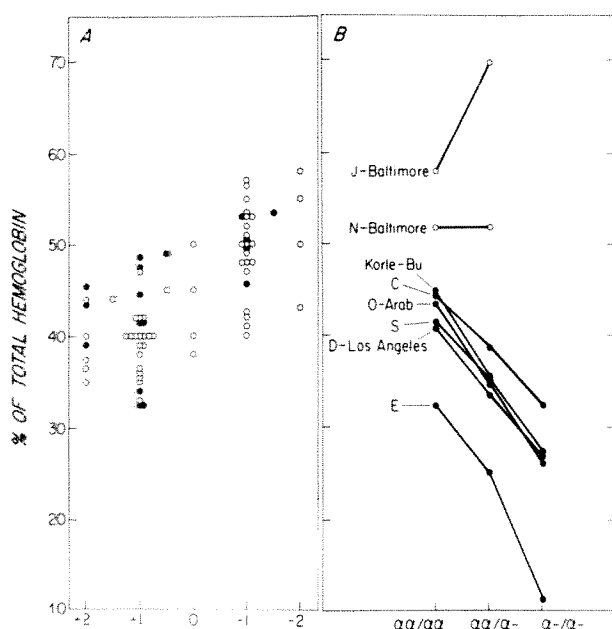


Fig 2. (A) Effect of charge on the proportion of abnormal hemoglobin in individuals heterozygous for 72 stable β -globin variants. Each data point represents a mean value for a given variant. The solid points (●) denote measurements of Huisman^{20a} using high-resolution chromatography. Substitutions involving a histidine residue were scored as a change of one half charge. The "-1" group differs significantly from the "+1" group ($P < .001$) and from the "0" group ($P < .05$). (B) Effect of α -thalassemia on the proportion of six positively charged β -chain variants (●) and two negatively charged variants (○) (data from Bunn and McDonald²⁰).

documented in the literature. Secondly, there is a twofold difference in the expression of the two tandem α -globin genes both at the mRNA level^{21,22} and at the protein level.²³ Both of these factors would confound interpretation of levels of α -globin mutants in heterozygotes.

These hematologic observations suggest that the surface charge of subunits is an important determinant of hemoglobin assembly. This hypothesis can be tested by preparing normal and variant subunits, mixing them, and monitoring the formation and dissociation of $\alpha\beta$ -dimers.

DISSOCIATION OF $\alpha\beta$ -DIMER INTO MONOMERS

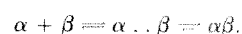
Until recently the combination of α - and β -monomers to form the $\alpha\beta$ -dimer was generally regarded as an irreversible reaction. Extreme, nonphysiologic solvent conditions were thought to be necessary to split the dimer into its constituent subunits. However, experiments measuring the displacement of α -subunits with radio-labeled α -subunits have shown conclusively that under physiologic conditions, the $\alpha\beta$ -dimer slowly dissociates into monomers.^{24,25} This first-order reaction is sensitive to temperature, pH, and ionic strength. Measurement of dimer dissociation has provided an explanation of a long-recognized and useful property of fetal hemoglobin: resistance to alkaline denaturation.²⁵ The $\alpha\gamma$ -dimer of Hb F dissociates about half as rapidly as that of Hb A at neutral pH. As pH is increased, the difference widens. The relatively slow rate of $\alpha\gamma$ dissociation at high pH compared

to that of $\alpha\beta$ dimer can be explained by the substitution of threonine for cysteine at position 112. This residue is one of the three sites at the intersubunit interface at which the γ - and β -subunits differ. It is likely that ionization of the sulfhydryl group of β 112 Cys at high pH contributes to subunit dissociation and subsequent denaturation of Hb A.²⁶

ASSOCIATION OF MONOMERS INTO $\alpha\beta$ -DIMERS

The rate-limiting step in hemoglobin assembly is the combination of heme-intact α - and β -globin subunits to form the $\alpha\beta$ -dimer. This reaction can be monitored by spectroscopic means to give a second-order rate constant of about $8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.^{27,28} This rate is nearly three orders of magnitude slower than the diffusion-limited rate for the combination of two macromolecules the size of globin subunits. Because of inherent inaccuracies in these spectroscopic measurements, we used subunit competition experiments to examine the assembly of hemoglobin variants and to test the effect of surface charge.²⁹ Previous experiments had shown that α -subunits combine about two times more readily with β^A than with β^S .³⁰⁻³³

Our subunit competition experiments were performed by incubating varying amounts of α -subunits with an equimolar amount of normal (β^A) and variant (β^X) β -subunits and then measuring the amounts of hemoglobin formed (Hb A and Hb X) by high-performance liquid chromatography and isoelectric focusing. Globin subunits were prepared with sufficient care that the addition of α -chains to an equivalent amount of β -chains resulted in the stoichiometric formation of hemoglobin tetramers having completely normal oxygen binding. These competition experiments closely simulate the *in vivo* situation in erythroblasts of heterozygotes where normal and variant β -subunits are competing for α -subunits. A low ratio of α to $\beta^A + \beta^X$ mimics the coexistence of α -thalassemia. At physiologic pH (7.2), the α -subunit has 2.4 positive charges, while the β^A subunit has 2.5 negative charges. Therefore, it makes sense that the two subunits would be pulled together by electrostatic attraction. This attraction would be altered if a variant β -subunit (β^X) differed significantly in surface charge from β^A . As shown in Fig 3, in the presence of limiting amounts of α -chains, the formation of Hb X relative to Hb A was directly related to the surface charge of the variant β -subunit. At pH 8.0, where α -subunits are at their isoelectric point and therefore lack significant net surface charge, differences in charge on the β -subunit no longer affect the rate of $\alpha\beta$ -dimer formation. These subunit competition experiments are in satisfactory agreement with a theoretical model based on the effect of smeared charge on the diffusion-limited association of macromolecules. As mentioned earlier, the overall rate of combination of α - and β -subunits is 1,000 times slower than the diffusion-limited reaction rate. Moreover, there is no significant difference in the rate of dissociation of $\alpha\beta$ -dimers of widely different charges ($\alpha\beta^N$, $\alpha\beta^A$, $\alpha\beta^C$). Taken together, these observations strongly suggest that hemoglobin assembles by the formation of an intermediate encounter complex ($\alpha \cdots \beta$) that can relax into the stable $\alpha\beta$ -dimer:



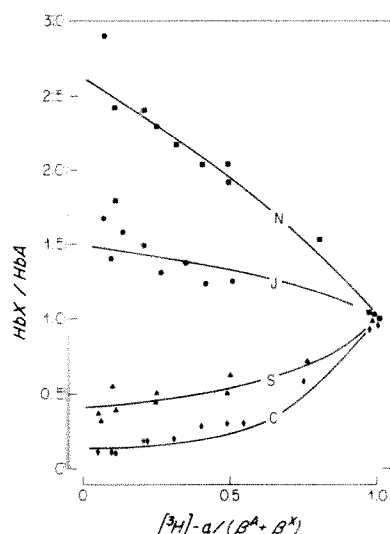


Fig 3. Subunit competition. Equimolar mixtures of normal (β^A) and a variant β -subunit (β^J , β^S , β^C) were mixed with increasing amounts of tritium-labeled α -subunits. At intracellular pH (7.2) these variant β -subunits in their native heme-intact state have net surface charges of -4.5 , -3.5 , -1.5 , and -0.5 , respectively. The products (Hb A and Hb X) were measured by high-performance liquid chromatography. The solid lines represent the computed theoretical fit for competitive recombination reactions between β^A and β^X , assuming that at low α -chain concentrations, the Hb X-Hb A ratio is a measure of the relative rates of assembly of $\alpha\beta^A$ and $\alpha\beta^X$ (data from Mrahet et al²⁹).

EFFECT OF SUBUNIT ASSEMBLY ON HEMOGLOBIN PHENOTYPE

This electrostatic model of hemoglobin assembly provides a sound explanation for why the levels of stable, positively charged β -globin variants in heterozygotes tend to be relatively low compared with Hb A and are further lowered in the presence of α -thalassemia. It also explains the converse observations on the negatively charged variants (see Fig 2). In addition, this model explains a number of other hematologic observations.

The pathophysiologic consequences of the sickling disorders are determined primarily by the concentration of Hb S in the patient's red cells.³⁴ Why do SC heterozygotes have a disease that is sometimes as severe as that of SS homozygotes, while AS heterozygotes are virtually free of clinical manifestations? There are two reasons why Hb S polymerizes more readily in SC than in AS red cells. First, because of potassium and water loss imposed by the presence of C hemoglobin,³⁵ SC red cells have a relatively higher concentration of total hemoglobin.^{36,37} Second, and of equal importance, the proportion of Hb S is 10% to 15% higher in SC red cells than in AS red cells.^{37,38} The latter finding can be explained by differences in the rates of subunit assembly. As shown in Fig 3, the relative rates of $\alpha\beta$ -dimer formation is $A > S > C$. These differences are reflected in the levels of hemoglobins in AS and SC red cells. The converse situation pertains when Hb S is inherited with a negatively charged β -globin variant. In these double heterozygotes, the proportion of Hb S is even lower, and thus the proportion of the variant is even higher than that found in association with Hb

A. For example, the proportion of Hb Pyrgos (β 83 Gly $\rightarrow$ Asp) was 62% in an S/Pyrgos double heterozygote, compared with 52% in relatives who were A/Pyrgos heterozygotes.³⁹ These findings are again fully consistent with the effect of surface charge on the assembly of normal and variant hemoglobins.

Measurement of minor hemoglobin components has been useful in the diagnosis of a number of hematologic disorders. Hb A₂ ($\alpha_2\delta_2$) normally constitutes about 2% of the hemolysate. This component is increased approximately twofold in β -thalassemia trait and decreased in various forms of α -thalassemia⁴⁰ as well as in two acquired disorders: iron deficiency^{40,41} and sideroblastic anemia.^{42,43} In the latter two conditions, a relative deficiency of α -globin synthesis has been noted in some studies.⁴³⁻⁴⁶ Therefore, they can be considered acquired forms of α -thalassemia. The δ -globin subunit is considerably more positively charged than is the β -subunit. Therefore, when the production of α -globin is limiting (α -thalassemia), the relative amount of Hb A₂ would be expected to decrease. In contrast, when α -subunits are present in excess, as in β -thalassemia, the small amount of δ -subunits that are produced should be fully titrated, and therefore Hb A₂ increases. This proposal is supported by competition experiments showing that α -subunits bind more avidly to β - than to δ -subunits.⁴⁷ The contribution of subunit competition to the formation of Hb A₂ provides a reasonable explanation for the slight elevation in Hb A₂ that has been noted in individuals with sickle trait (AS)⁴⁸ as well as in SS homozygotes who also have α -thalassemia.⁴⁹

Hb F ($\alpha_2\gamma_2$), the major hemoglobin component of the fetus, is normally replaced by Hb A by means of a switching mechanism that takes place during the latter part of gestation. Whatever Hb F that remains is compartmentalized in a restricted population of red cells. Elevations of Hb F in a variety of congenital and acquired disorders are generally proportional to the number of F cells that are produced. However, levels of Hb F could also be affected by differences in assembly. Because β - and γ -subunits differ considerably in primary structure (39 out of 146 residues), it is likely that factors other than charge affect their relative rates of binding to α -subunits. Because native heme-intact γ -subunits are difficult to isolate and work with, no competition experiments have been done. However, some recent clinical observations address this issue. In newborns, the proportion of Hb F is lower in those with α -thalassemia than in those having all four α -globin genes.⁵⁰ In addition, an individual heterozygous for deletion-type hereditary persistence of fetal hemoglobin was found to have only 7% Hb F when he had severe iron deficiency.⁵¹ Iron replacement resulted in increased synthesis of α -globin subunits as well as an increase in Hb F to a steady-state level of 25%. These hematologic observations suggest that α -subunits combine less readily with γ - than with β -subunits.

In keeping with this model of hemoglobin assembly are the observations that when AS and AE heterozygotes become iron deficient, the level of the positively charged variant hemoglobin drops markedly^{52,53} but, as with the patient described earlier, is readily corrected by iron replacement. It is likely in all these cases that the acquired α -thalassemia

intensifies the competition between normal and the variant subunit.

Although a large body of hematologic and experimental observations supports this model certain exceptions can be cited. For example, the level of Hb C in AC individuals is about the same as that of Hb S in those with sickle trait (Fig 2). Moreover, α -thalassemia has no effect on the proportion of S and C hemoglobin in individuals with SC decrease.⁵⁴ Thus, in vivo β^S and β^C compete about equally well for α -subunits, whereas in vitro (Fig 3) β^S combines about three times as readily as β^C . It is possible that mild instability of Hb S⁵⁵ is an independent contributor to the proportion of Hb S in circulating red cells.

TARGET CELLS

The surface charge of hemoglobin may impact on another phenotypic feature: red cell volume. One of the most commonly observed characteristics of "hemoglobinopathies" is the presence of target cells on dried blood films. This finding is accompanied by a shift to the right of the osmotic fragility curve indicative of a relative decrease in the ratio of cell volume to surface area. Target cells are prominent in the following commonly encountered genotypes: CC, AC, SS, EE, AE, and DD. In a rare homozygote with hemoglobin O-Arab, both target cells and a right-shifted osmotic fragility were noted.⁵⁶ All of these variants are positively charged. There is solid experimental evidence that hemoglobin binds to the inner surface of the red cell membrane by electrostatic interactions,⁵⁷⁻⁵⁹ probably to a negatively charged domain on the cytoplasmic side of the major integral membrane protein, Band 3. Therefore, it is likely that this binding is affected by

the surface charge of hemoglobin. Hb C binds more strongly than Hb A to the cytoplasmic surface of red cell membranes.⁶⁰ This "assembly" may be an integral part of a recently described pathway that causes leakage of potassium and water from red cells.³³ This pH-and-ionic strength-dependent pathway is active in CC red cells and is also readily observed in AC, SS,⁶¹ and EE red cells. Thus, the surface charge of hemoglobin may be a critical determinant of red cell volume and may explain the presence of cell dehydration and target cells that is such a prominent feature of the common positively charged variants.

BROADER IMPLICATIONS

The assembly of hemoglobin is apt to be relatively simple compared with that of other biologically important macromolecules. An array of biophysical probes is becoming available to investigate these important posttranslational events. Examples of particular interest to hematologists include (a) secreted proteins, such as immunoglobulin and factor VIII-von Willebrand factor; (b) membrane complexes, such as the T cell receptor for antigen and platelet membrane glycoproteins 2B-3A; (c) structural proteins such as the spectrin-actin-4.1 assembly in the red cell cytoskeleton; and (d) the complex variety of protein-DNA and protein-RNA interactions that regulate gene expression.

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Evidence for Chemical Differences in HLA-DR Molecules on Autologous Acute Lymphoblastic Leukemia and B-Lymphoblastoid Cell Lines

By Lynn Graf and John M. Pesando

HLA-DR molecules on autologous acute lymphoblastic leukemia (ALL) and B-lymphoblastoid cell lines from two individuals were compared by immune precipitation and gel electrophoresis. Cells were surface labeled with ^{125}I and proteins immunoprecipitated with specific monoclonal antibodies (MoAb). Two electrophoretically distinct bands were found in the HLA-DR beta chain region on both ALL cell lines in contrast to only one on each of the autologous B-lymphoblastoid cell lines. Differences in the electrophoretic mobility of the alpha chains were also observed with ALL and B-lymphoblastoid cell lines from one individual. Preclearing of radiolabeled cell lysates with MoAb specific for HLA-DQ and -DP molecules demonstrated that the complexity of the HLA-DR pattern is not the result of

antibody cross-reactivity with alpha and beta chains from other class II products. Immunoprecipitation experiments indicated that two beta chain bands are observed with each of the parental HLA-DR molecules on the ALL but not the B-lymphoblastoid cell line from an HLA-DR3,7-positive individual. We conclude that the HLA-DR molecules expressed on ALL and B-lymphoblastoid cell lines from the same individual can differ chemically. Neuraminidase treatment reduced these electrophoretic differences, indicating that these molecules differ in their sialic acid content. Since small changes in class II molecules can profoundly alter cellular interactions, the functional significance of these differences requires further investigation.

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CLASS I AND II MOLECULES of the major histocompatibility complex (MHC) serve as corecognition elements in antigen presentation to T lymphocytes. Small differences in these cell surface glycoproteins can profoundly affect their ability to mediate interactions among cells involved in the immune response.¹ Recent studies indicate that alterations in these molecules contribute to malignant behavior by interfering with the recognition of tumor cells by the immune system of the host.²⁻⁴

Class II molecules are polymorphic heterodimers comprised of heavy (alpha) and light (beta) chains having molecular weights of 33 to 35 kilodaltons (kd) and 26 to 31 kd, respectively. In an extensive series of experiments using indirect immunofluorescence and immunoprecipitation assays, we have previously shown that malignant B cells often fail to express one or more of the three types of class II molecules (HLA-DR, -DQ, and -DP) present on normal B lymphocytes.⁵ We now demonstrate by immunoprecipitation and gel electrophoresis that serologically identical HLA-DR molecules expressed on cell lines derived from autologous normal and malignant B cells from two individuals with acute lymphoblastic leukemia (ALL) are chemically distinct. These HLA-DR molecules differ in their sialic acid content, although we cannot rule out additional chemical differences at this time.

MATERIALS AND METHODS

Cell lines and antibodies. The autologous ALL (Laz 221 and NALM-6) and Epstein-Barr virus (EBV)-transformed normal B cell lines (Laz 388 and B85) have been described previously.⁵⁻⁷ The autologous nature of each pair of cell lines was confirmed by HLA typing with alloantisera by Dr Patrick Beatty of the Puget Sound Blood Center. Laz 221 and Laz 388 cells are HLA-DR1, DR5-, and DRw52-positive, while NALM-6 and B85 are DR3-, DR7-, and DRw52-positive. All cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Class II molecules were identified by the following monoclonal antibodies (MoAb): HLA-DR (p4.1,⁸ I-2,⁹ ISCR3,¹⁰ 1-32, 7.2,¹¹ and SFR16-DR7M¹²); HLA-DQ (Leu 10,¹³ Genox 3.53,¹⁴ and 1-296); and HLA-DP (B7/21¹⁵) (specificities as discussed in Pesando and Graf⁵).

Cell surface labeling, immunoprecipitation, and gel electrophoresis. Cells were surface-labeled with ^{125}I (1 mCi/25 × 10⁶ cells)

using the lactoperoxidase method⁹ and lysed with Tris-HCl-buffered Triton X-100 (Sigma, St Louis). MoAb as hybridoma supernatants or ascites fluid were adsorbed to the appropriate matrix (Protein A-Sepharose, Pharmacia, Piscataway, NJ, or rabbit anti-mouse Ig Sepharose) overnight prior to addition of radiolabeled lysate. Class II molecules were immunoprecipitated and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 25-cm 10%- to 15%-gradient slab gels according to the method of Dobberstein et al.¹⁶ For sequential immunoprecipitations, lysate was first depleted of one antigen by four treatments with the same MoAb before being used for immunoprecipitation with additional MoAb.

Where indicated, immunoprecipitates were treated four times with 0.025U neuraminidase (*Vibrio cholerae*, Calbiochem, San Diego) over a period of six hours at 37 °C in 0.1 mol/L acetate buffer, pH 5.5, containing 9 mmol/L CaCl₂ prior to two-dimensional gel electrophoresis. Washed immunoprecipitates were separated by SDS-PAGE as described above. HLA-DR molecules were located with the aid of fluorescent marker proteins, excised, and analyzed by isoelectric focussing (IEF) at pH 5 to 8 as described by Shackelford and Strominger.¹⁷

RESULTS

Molecules identified by HLA-DR-, -DQ-, and -DP-specific MoAb were precipitated from surface-iodinated

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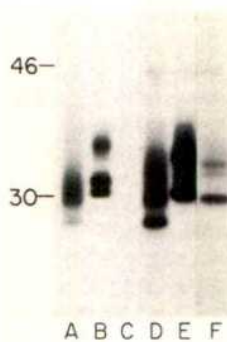


Fig 1. Comparison of class II molecules on autologous ALL and B-lymphoblastoid cell lines by SDS-PAGE. The class II molecules from Laz 221 (lanes A through C) and Laz 388 (lanes D through F) cells were immunoprecipitated with the HLA-DP-specific MoAb B7/21 (lanes A and D), the HLA-DR-specific MoAb p4.1 (lanes B and E), and HLA-DQ-reactive MoAb Genox 3.53 (lanes C and F). 2×10^6 cell equivalents were used for each immunoprecipitation, and the autoradiogram was exposed 1 week. The varying intensity of these bands does not necessarily reflect the number of molecules expressed because of differences in the efficiency of iodination of different cell lines and molecules. Approximate molecular weights in kilodaltons are indicated on the left.

autologous ALL and B-lymphoblastoid cell lines (Figs 1 and 3). HLA-DR and -DP molecules were detected on both ALL and B-lymphoblastoid cell lines, whereas HLA-DQ molecules were detected only on B-lymphoblastoid cells, in agreement with our previous data.⁵ SDS-PAGE analysis of molecules on Laz 221 and Laz 388 cells (Fig 1) demonstrated the presence of two electrophoretically distinct HLA-DR beta chain bands of equal intensity on the ALL cell line versus a single beta chain on the autologous B-lymphoblastoid one. Side-by-side comparison also revealed slight differences in the electrophoretic mobility of the respective alpha chains. Four additional HLA-DR-specific MoAb (I-2, 1-32, 7.2, and ISCR3) produced identical results with these autologous cell lines (data not shown). Thus it is unlikely that the additional HLA-DR beta chain band is a protein that was nonspecifically coprecipitated by MoAb p4.1. Neither the position nor the intensity of any of the HLA-DR bands was altered by exhaustive preclearing of the radiolabeled lysate with MoAb specific for either HLA-DQ (Genox 3.53) or HLA-DP (B7/21) molecules, demonstrating that cross-reactivity of HLA-DR antibodies with other class II products was not responsible for the complexity of the pattern of HLA-DR molecules on the ALL cells (Fig 2). There may also be slight differences in the higher molecular-weight region of the HLA-DP molecules radiolabeled on these two cell lines, but the poor resolution of HLA-DP beta chain(s) on Laz 221, a consistent finding with this cell line, did not permit an exact comparison. HLA-DP alpha chains are not radiolabeled by surface iodination.¹⁵

Figure 3 shows a similar comparison of a second ALL cell line, NALM-6, with its autologous B-lymphoblastoid cell line B85. HLA-DR-specific MoAb again immunoprecipitated what appear to be two distinct bands in the beta chain region in the ALL cells, with the upper band being fainter

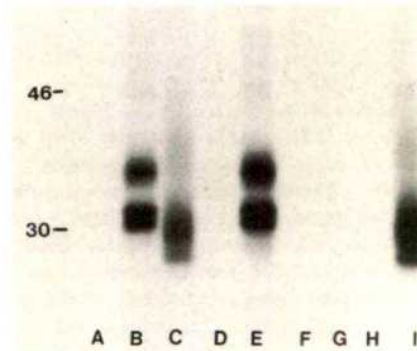


Fig 2. Sequential immunoprecipitations with anti-class II MoAb. Lysates from surface-iodinated Laz 221 cells were precleared in parallel with MoAb specific for HLA-DQ (Genox 3.53, lanes A through C), HLA-DP (B7/21, lanes D through F) or HLA-DR (p4.1, lanes G through I) and split into three aliquots which were used for immunoprecipitation with the same three antibodies. Lanes A, D, and G: Genox 3.53; lanes B, E, and H: p4.1; lanes C, F, and I: B7/21.

than the lower one. A shorter exposure of this autoradiograph (data not shown) confirmed the appearance of only a single HLA-DR beta chain band on the B85 cell line (lanes F and G).

Both pairs of autologous cell lines are heterozygous for HLA-DR molecules (see Materials and Methods). Reactivity of alloantisera indicate that NALM-6 and B85 are both HLA-DR3,7-positive. To determine the contributions of these two parental HLA-DR haplotypes to the observed electrophoretic patterns, we isolated and studied these molecules using the polymorphic HLA-DR-specific MoAb I-2 and SFR16-DR7M. I-2 reacts with cells of all HLA-DR types except DR7.⁹ Thus it should only react with HLA-DR3 molecules on NALM-6 and B85. In contrast, SFR16-DR7M only binds to molecules that carry the HLA-DR7 serologic specificity.¹² Immunoprecipitation with these two MoAb (Fig 4) indicated that HLA-DR3 and -DR7 molecules are expressed on both of these cell lines. Moreover, the beta chains of both the HLA-DR3 and -DR7 molecules appeared

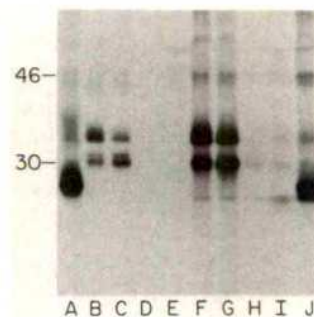


Fig 3. Class II molecules on the NALM-6 (lanes A through E) and B85 (lanes F through J) cell lines were detected by immunoprecipitation with MoAb B7/21 (lanes A and J); HLA-DR-specific MoAb p4.1 (lanes B and F) and I-2 (lanes C and G); and HLA-DQ-reactive MoAb Leu 10 (lanes D and H) and 1-296 (lanes E and I).

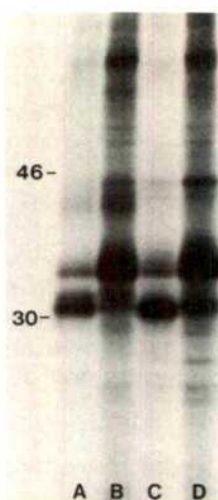


Fig 4. Analysis of HLA-DR molecules immunoprecipitated from surface-iodinated NALM-6 (lanes A and B) and B85 (lanes C and D) cells by polymorphic MoAb. MoAb I-2, which reacts only with DR3 molecules in these cells, was used for lanes A and C. The DR7-specific MoAb SRF16-DR7M was used for samples in lanes B and D.

as doublets in the ALL cell line and as singlets in the B-lymphoblastoid line.

Since both the alpha and beta chains of HLA-DR molecules are glycosylated,¹⁷ we evaluated the possible role of sialic acid residues in producing the apparent molecular-weight differences and doublet patterns observed by SDS-PAGE. Immunoprecipitates from all four cell lines were treated with neuraminidase (0.1 U total) or sham-treated and analyzed by SDS-PAGE (Fig 5). Treatment with neuraminidase reduced the apparent molecular weight of both the alpha and beta chain(s) on all cell lines, but some differences remained between the HLA-DR molecules from autologous cell populations. Treatment of the sample shown in lane E with twice the standard amount of neuraminidase produced no further changes detectable in this gel system.

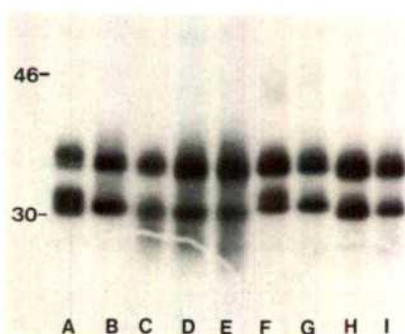


Fig 5. Effect of neuraminidase treatment. HLA-DR molecules were immunoprecipitated with the HLA-DR-specific MoAb p4.1 from Laz 221 (A and C), Laz 388 (B, D, and E), NALM-6 (F and H) and B85 (G and I) cell lines. Samples were sham-treated (A, B, F, and G) or treated with 0.1 U neuraminidase (C, D, H, and I). Sample E from Laz 388 cells was incubated with 0.2 U neuraminidase.

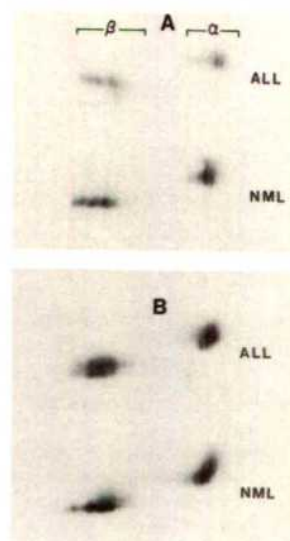


Fig 6. Two-dimensional gel electrophoresis of HLA-DR molecules immunoprecipitated from Laz 221 (ALL) and Laz 388 (NML) cells with MoAb ISCR3. The samples with (A) or without (B) neuraminidase treatment were separated by SDS-PAGE, then analyzed on an isoelectric focussing gel, pH 5 to 8. Brackets indicate the approximate position of alpha and beta chains. The acidic pole is to the right and the basic pole to the left.

HLA-DR molecules from the Laz 221 and Laz 388 cell lines (DR1,5; DRw52) were also analyzed with and without neuraminidase treatment by two-dimensional gel electrophoresis according to the method of Shackelford and Strominger¹⁸ (Fig 6). The MoAb used (ISCR3) has been shown to react with both the DR1 and DR5 haplotypes. Two rows of spots in the beta chain region, one above the other and with the upper set shifted slightly to the acidic pole, were observed in samples of the ALL line that were processed without neuraminidase treatment (Fig 6B). Only a single row of spots was present in HLA-DR molecules from corresponding samples from the B-lymphoblastoid cells. The desialylated HLA-DR beta chains on these autologous cell lines (Fig 6A) appeared to be quite similar.

HLA-DR molecules immunoprecipitated from NALM-6 and B85 cells with the I-2 and SFR16-DR7M MoAb were also treated with neuraminidase and analyzed by two dimensional gel electrophoresis (Fig 7). The HLA-DR7 beta chain spots are more acidic than those from HLA-DR3 molecules. This confirmed that the two MoAb react with different HLA-DR molecules. The desialylated HLA-DR beta chains on the autologous ALL and B-lymphoblastoid cell lines again appeared to be quite similar. Minor differences remaining could result either from incomplete removal of sialic acid residues or from further as yet unidentified glycosylation differences.

DISCUSSION

These electrophoresis studies document chemical differences in the HLA-DR molecules expressed on autologous malignant versus EBV-transformed B cell lines from two individuals with ALL. The most significant finding is the presence of two electrophoretically distinct bands in the beta

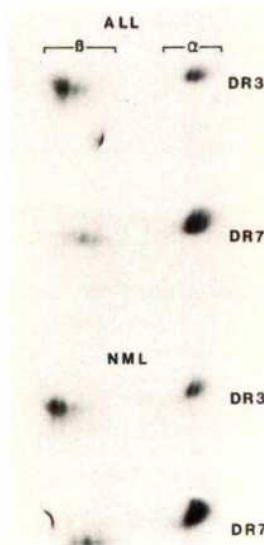


Fig 7. Two-dimensional gel electrophoresis of neuraminidase treated HLA-DR molecules from NALM-6 (ALL) and B85 (NML) cells. The HLA-DR3-specific MoAb I-2 and the HLA-DR7-specific MoAb SFR16-DR7M were used to study both of these cell lines. All four samples were compared simultaneously as in Fig 6.

chain region of HLA-DR molecules from ALL but not B-lymphoblastoid cell lines. This pattern is observed with multiple monomorphic HLA-DR-specific MoAb. Use of polymorphic HLA-DR-specific MoAb indicates that two beta chain bands are found with both of the parental HLA-DR molecules on ALL cells from an HLA-DR 3,7-positive individual. We cannot yet determine what role, if any, HLA-DR beta chains carrying the alloantiserum-defined DRw52 specificity play in the electrophoretic patterns observed. Immunodepletion studies employing HLA-DQ- and -DP-specific MoAb indicate that the unique appearance of HLA-DR molecules on ALL cells is not due to serologic cross-reactivity with the alpha or beta chains of other class II molecules. While we do not know how closely the HLA-DR molecules on the two types of B cell lines resemble those of normal and malignant B cells occurring in vivo, this is the first report describing differences in the HLA-DR molecules on autologous cells within the same lineage.

If the genes encoding class II molecules are identical in normal and leukemic B cells from the same individual, then differences in individual molecules expressed on the cell surface most likely reflect unique post-translational modifications of common precursor proteins or variable surface expression of shared molecules. Our studies indicate that the HLA-DR molecules from these two cell types differ in their sialic acid content. Whether additional glycosylation differences also play a role in the observed unique electrophoretic patterns is unclear. Molecular weight variations between the alpha chains of HLA-DR molecules on a human melanoma line and its autologous B-lymphoblastoid cell line were found to reflect unique carbohydrate structures including but not limited to sialic acid.¹⁹

Small changes in class II molecules can have profound effects on the process of self-recognition. Thus the spontaneous bm12 mutant of the mouse demonstrates mixed lymphocyte reactivity with cells of the parent strain, a new pattern of MHC-restricted antigen presentation, and altered binding of class II-specific MoAb.²⁰ The class II molecules of the parent and mutant strains differ by only three amino acids.²¹ Single amino acid substitutions in murine class II molecules can similarly alter their capacity to present antigens to T lymphocytes.^{1,22,23} Cowing et al²⁴⁻²⁶ report that differences in the carbohydrate moieties on class II molecules can also affect their function. The increased number of sialic acid residues on class II molecules from resting B lymphocytes versus accessory cells from mouse spleen appears to impair the ability of the former cells to activate T lymphocytes. Treatment of splenic B cells with neuraminidase to remove sialic acid residues restores their ability to trigger T cell proliferation. These studies suggest that the chemical differences that we have observed in the HLA-DR molecules on autologous normal and malignant human B cell lines might be functionally significant. Preliminary experiments in our laboratory indicate that these autologous cell lines differ in their ability to present cell-associated antigens to T lymphocytes.

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Loss of Interleukin-2 Requirement for the Generation of T Colonies Defines an Early Event of Human T-Lymphotropic Virus Type I Infection

By Madeleine Duc Dodon and Louis Gazzolo

Accessory cells and/or soluble factors, together with interleukin 2 (IL2), are required for the proliferation and differentiation of phytohemagglutinin (PHA)-activated T lymphocytes. Human T-lymphotropic virus, type I (HTLV-I), a human retrovirus isolated from patients with adult T cell leukemia, can transform T cells in vitro. We investigated the role of HTLV-I-transformed T cell lines as accessory cells in promoting the growth of T colony-forming cells. We found that T cells isolated by E rosetting and then activated with PHA, when seeded with as few as 5×10^3 irradiated HTLV-I-producing cells, could generate colonies in the absence of IL2. We analyzed further the effects of HTLV-I virions on T colony formation. Infection of T cells with semipurified HTLV-I viral particles promoted colony formation, in the absence of IL2, of accessory cells or soluble factors. The same results were obtained either

with monocyte-depleted T lymphocytes, or with T4 or T8 lymphocytes. Furthermore, T lymphocytes in the presence of heat-inactivated HTLV-I (devoid of replicative potential) could form colonies independently of IL2. Finally, experiments with sera positive for HTLV-I antibodies (to abolish binding of viral particles to cellular receptors) indicated that HTLV-I promoted IL2-independent colony formation, only by "touching" T colony-forming cells. These results taken together demonstrate that the loss of the exogenous IL2 (and other growth-helping factors) requirement defines an early event of HTLV-I infection. The results also suggest that viral attachment to T cells possibly supplies an accessory function triggering autocrine secretion of IL2 by these cells.

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THE IDENTIFICATION, isolation and characterization of growth factors have provided new and challenging insights on the mechanisms governing cellular proliferation and differentiation. In particular, lymphokines produced by antigen- or mitogen-activated T lymphocytes are responsible for the multiple effects of cellular immune reactions, by regulating the differentiation of lymphoid cells. Among these lymphokines, IL2 (formerly known as T cell growth factor¹) is required for the amplification of T cell populations. Upon exposure to antigens, lectins and other mitogenic signals, T lymphocytes express cell surface IL2 receptors.² Such activated T cells are now able to produce IL2 which, after interaction with specific high-affinity IL2 receptors, stimulates the growth of these cells.^{3,4} Furthermore, the proliferative response of T cells to activating agents is effective only in the presence of accessory cells.

Studies on the generation of colonies by T cells have provided much valuable information on the parameters controlling T cell proliferation in vitro.⁵⁻⁸ Thus, IL2 and accessory cells are both required for PHA-induced T colony formation. Accessory cells, which include monocytes, T cells, and B cells of normal and leukemic origin, are implicated both in the induction of IL2 synthesis and in the expression of functional IL2 receptors allowing IL2 responsiveness by the

T colony-forming cells.⁸ The same colony-forming ability was also observed when soluble factors released by accessory cells were incorporated in the colony medium.⁶

Because it leads to the amplification of activated T cells, IL2 has been decisive in isolating and characterizing human T-lymphotropic retroviruses (HTLV),⁹ such as HTLV-I associated with T cell leukemia (ATL) and LAV (lymphadenopathy-associated virus)/HTLV-III associated with the acquired immunodeficiency syndrome.^{10,11}

HTLV-I is able to transform T cells in vitro. T lymphocytes obtained from peripheral blood, umbilical cord blood or bone marrow and infected with HTLV-I are in most cases grown in the presence of IL2 until the overgrowth by transformed T cells which have become independent on this growth factor, several weeks after infection.^{12,13} T cell lines derived from leukemic donors and T cell lines obtained after in vitro infection both continue to express IL2 receptors. Moreover, the majority of these HTLV-I-transformed cell lines do not release IL2, while they are still able to secrete a large variety of other lymphokines.¹⁴

Several studies have shown that infection of T cells by HTLV-I causes impaired immune functions of T cells.¹⁵ Once infected, these cells might also disturb the growth of normal T cells. Preliminary observations on the role of HTLV-I transformed T cell lines as accessory cells led us to investigate further the involvement of viral particles in T colony formation. The results reported in this paper indicate that HTLV-I infected cells and HTLV-I virions are both able to induce the proliferation of T colony-forming cells in the absence of exogenous IL2.

MATERIALS AND METHODS

Cell separation. Peripheral blood was collected from normal healthy volunteers. Leukocytes (PBL) were obtained after buffy coat sedimentation at unit gravity for 45 minutes using a 1% methylcellulose solution. Mononuclear cells (MNC) were separated by Ficoll-Hypaque density centrifugation ($d = 1.077 \text{ g/mL}$), washed twice with RPMI 1640 and resuspended in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS). T cell separation was carried out according to Madsen et al¹⁶ by rosetting with 2-aminoethylisothiuronium bromide (AET)-treated sheep red

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blood cells (SRBC) and a second density centrifugation was performed through Ficoll-Hypaque. The E rosette-depleted cells (E^-) were collected from the interface, and the E rosette-enriched cells (E^+) were recovered from the pellet, after hypotonic lysis of the SRBC in 0.15 mol/L ammonium chloride. The E^+ cell population contained a majority of T cells (over 95%), as assessed by immunofluorescent labeling with OKT3 monoclonal antibody, and about 5% of nonspecific esterase-positive monocytes and of surface Ig-positive cells.

In specific experiments, purified T lymphocytes were obtained by using a three-step procedure. MNC were depleted of adherent cells by one-hour incubation in a plastic culture flask, followed by the elimination of phagocytic cells by carbonyl iron ingestion at 37 °C for one hour and passage over a magnet. T lymphocytes were then isolated by overnight incubation with AET-treated SRBC and centrifugation over a Ficoll-Hypaque gradient. Almost pure T cells (OKT3-positive) were recovered from the pellet after lysis of the SRBC. These T cells were further separated in T4 or T8 lymphocytes by treatment with the OKT8 or OKT4 monoclonal antibodies, respectively, and then appropriately diluted newborn rabbit complement, with the use of a cytotoxic assay.

Monocytes were prepared after adherence of MNC to plastic culture flasks. After elimination of the nonadherent cells and extensive washings, adherent cells were detected after a 20-minute incubation in 3 mmol/L EDTA. This cell population was found to contain more than 95% nonspecific esterase-positive monocytes and less than 3% OKT3 cells.

T colony-forming assay. The T colony assay was performed according to a previously described technique.⁸ Cultures were prepared in 35-mm plastic Petri dishes, by using two layers of semisolid agar medium of 1 mL each. The underlayer contained 0.45% agar, 10% of a mixture of human AB serum and FCS, 40 μ g of phytohemagglutinin (PHA)-P (Wellcome) and 10% lectin-free IL2 (Cellular Products) in α -minimum essential medium (MEM). This was overlaid with 1 mL of 0.30% agar in α -MEM containing 2×10^5 E^+ cells, 1×10^6 X-irradiated accessory cells (PBL or E^- cells), or conditioned medium from Raji cells, to a final concentration of 40%. Plates were then incubated for seven days at 37 °C in 5% CO₂-humidified atmosphere. On days 1 through 3, colonies were not generally found. On days 4 to 5 after seeding, an increase in the number of colonies was observed. This number reached a peak on day 7 or 8 at which time they were counted using a Nikon inverted microscope. Only clusters containing more than 40 cells were scored as colonies. Each assay was performed in duplicate. The results of at least five separate experiments were expressed as mean $\pm$ SD.

Cell lines. All cell lines (kindly provided by Dr R.C. Gallo, Bethesda, MD) used in this study were grown in RPMI 1640 supplemented with 10% FCS. The HTLV-I-transformed T cell lines included HUT102¹⁷ (derived from a patient with a cutaneous T cell lymphoma) MT2,¹⁸ C91/PL,¹⁹ and C81-66/45,²⁰ all obtained after transformation of cord blood cells, cocultivated with HTLV-I-producing cells. All these transformed HTLV-I T cell lines express the 42-kilodalton HTLV-I *x-lor* protein product correlated with *trans*-activation of the long terminal repeat.^{21,22} However, the C81-66/45 cell line is nonproductively infected with HTLV-I, since these cells do not express *gag* antigens, and reverse transcriptase activity and viral particles were not detected in culture medium.²⁰ Finally, the H9 clone of the HT lymphoblastoid cell line was also used in this study, together with LAV/HTLV-III-infected H9 cells.¹¹ Before addition to the upper layer with E^+ cells, these cell lines were extensively washed, resuspended in medium and X-irradiated at 6,000 R.

The conditioned medium from Raji cells (a B cell line) was obtained by cultivating these cells in RPMI 1640 supplemented with 10% FCS for four days at 37 °C. The supernatant was then collected,

pooled after centrifugation at 1,000 g for ten minutes and filtered through a 0.45 μ m membrane filter. It was stored at -20 °C.

Viral infection of cells. Media containing either HTLV-I or LAV/HTLV-III particles were harvested from the different cells listed above. Following removal of the cells by low-speed centrifugation, the virus containing supernatant was concentrated 20-fold by centrifuging for three hours at 32,000 g in a Beckman type 35 rotor and resuspending the viral pellets in an appropriate volume of RPMI 1640 supplemented with 10% FCS. The virus was frozen at -70 °C, until infection of E^+ cells: 100 μ L of viral suspension and 100 μ L of E^+ cell suspension (at a concentration of 4×10^6 cells per mL) were mixed in a glass tube and incubated at room temperature for 30 minutes. At the end of the incubation period, cells were added to 1,800 μ L of agar medium and delivered into two 35-mm dishes, on the top of the hard agar layer. The virus was heat-inactivated by incubation at 56 °C for one hour, just before infection of E^+ cells.

Sera to be used in neutralization assays were heat-inactivated at 56 °C for 30 minutes. For each assay, one volume of viral suspension and one volume of serum at the desired dilution were mixed and incubated for one hour at room temperature, just before infection of E^+ cells.

RESULTS

Effect of HTLV-I-producing lines on T cell colony formation. As indicated in Materials and Methods, the presence of accessory cells or that of soluble factors produced by them was found to be mandatory for colony formation by E^+ cells under our experimental conditions. No colony formation was observed when E^- cells were incubated under the same assay conditions.

Experiments to ascertain the role of HTLV-I-producing T cell lines as accessory cells in T colony formation were performed in the absence or in the presence of IL2. In fact, some of the HTLV-I-transformed cells (MT2, HUT102) were found to secrete low amounts of IL2²³ (data not shown). As indicated in Table 1, it was surprising to find that IL2 was not necessary for colony formation when irradiated HTLV-I-producing cells were seeded in the upper layer. In fact, T colony-forming cells proliferated when as few as 5×10^3

Table 1. Effect of HTLV-I-Transformed T Cell Lines as Accessory Cells on the Number of Colonies Formed by E^+ Cells, in the Absence of IL2

Irradiated Accessory Cells	HTLV-I Production	Number of Cells	Number of Colonies/Plate
Transformed with HTLV-I			
C91/PL	+	5 × 10 ³	1312 ± 187
		20 × 10 ³	1474 ± 225
MT2	+	5 × 10 ³	2030 ± 155
		20 × 10 ³	TMTC
HUT102	+	5 × 10 ³	1225 ± 72
		20 × 10 ³	1737 ± 75
C81-66/45	—	5 × 10 ³	0
		20 × 10 ³	0
Not transformed with HTLV-I			
H9	—	5 × 10 ³	0
		20 × 10 ³	0
Peripheral blood leucocytes	—	5 × 10 ³	0
		20 × 10 ³	0

Accessory cells were X-irradiated at 6,000 R and then seeded in the agar upper layer together with 2×10^5 E^+ cells. Data expressed as mean $\pm$ SD. TMTC, too many to count.

irradiated cells were cocultured with them. With the same number of irradiated normal PBL, colony formation was not observed. The majority of colonies obtained in the presence of irradiated HTLV-I-transformed cells were larger and contained more than 100 cells. That explains why in some cultures T colonies were too numerous to count when 20×10^3 irradiated transformed cells were seeded. In addition, overgrowth of the cultures in some dishes did not favor the development of colonies. The same was true when IL2 was added to these cultures (data not shown).

Only cells transformed by and producing HTLV-I were able to cooperate in the colony growth. The lymphoblastoid T cells (H9, not infected by HTLV-I) and C81-66/45 cells (which are transformed by HTLV-I but defective in the replication of the virus) did not promote colony formation when seeded at 5×10^3 cells/dish. These results suggest that generation of T colonies in the presence of HTLV-I-producing cells, but in the absence of exogenous IL2, may be accounted for by viral particles and/or viral antigens released by these cells.

HTLV-I infection induces T colony formation in the absence of IL2 and monocytes. To test this hypothesis, virus was concentrated from the culture supernatant of one HTLV-I-producing cell line (C91/PL). One volume of the viral preparation was added to one volume of the E⁺ cell suspension. After a 30-minute incubation at room temperature, cells were seeded into the agar upper layer at three different concentrations. The results of a representative experiment are shown in Fig 1. Under standard conditions in which HTLV-I-treated E⁺ cells were seeded at 2×10^5 cells/dish, T cell colony formation was observed in the absence of added IL2. No colony was detected in virus-untreated cultures (control) seeded at this concentration. At lower concentrations of HTLV-I-infected E⁺ cells (1×10^5 cells/dish), there was no promotion of colony growth in the absence of IL2. Only in control E⁺ cultures with more than 3×10^5 cells/dish were few colonies detected when IL2 was

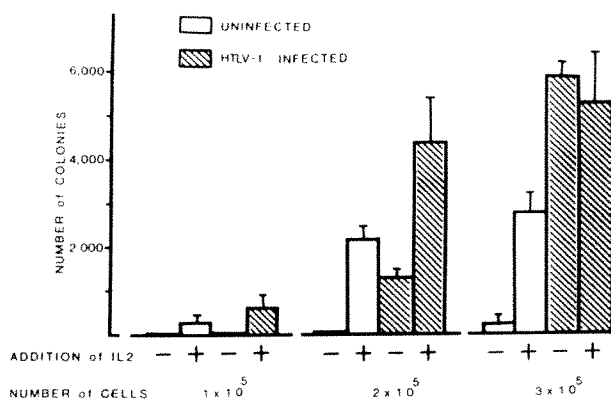


Fig 1. T colony formation by uninfected E⁺ cells and E⁺ cells infected by HTLV-I (harvested from the medium of C91/PL cells, and concentrated 20 times). E⁺ cells were seeded in the agar upper layer, at three different cell concentrations, in the presence or absence of IL2. PHA (40 μ g/mL) and IL2 (1 U/mL) were added in the agar underlayer. Raji conditioned medium was added in the agar upper layer, at a final concentration of 40%. Colony numbers are expressed as mean $\pm$ SD.

omitted. This low number of colonies may reflect the endogenous production of IL2. It should be noted that at this latter cell density (3×10^5), in the absence of exogenous IL2, colonies in HTLV-I-treated cultures were 30 times more numerous than those found in control cultures (6,000 to 200, respectively).

When IL2 was added in the medium, colonies were observed with control as well as with virus-treated cells. However, the number of colonies in HTLV-I-treated cultures was about twofold higher than that in control cultures. Once again, colonies observed in E⁺ cultures incubated with virus in the absence of IL2 were larger than those in control cultures, grown in the presence of IL2. In the absence of PHA, HTLV-I-treated T lymphocytes as well as control T lymphocytes failed to generate colonies.

Further results shown in Fig 2 indicate that (1) no modification in the colony growth was noted when the virus-treated cells were seeded in the presence or absence of conditioned medium (Fig 2A); (2) the number of colonies obtained was proportional to the amount of virus added (Fig 2B); no colony formation could be demonstrated in the virus-free supernatant (obtained after high-speed centrifugation of the C91/PL growth medium (Fig 2C); (3) HTLV-I viral particles concentrated from the culture fluid of other HTLV-I-producing cell lines induced colony formation; conversely, LAV/HTLV-III was unable to promote colony formation without IL2 (Fig 2D); after addition of IL2, the same number of colonies as in the control cultures was observed with LAV/HTLV-III infected T cells (data not shown).

Phenotypic characterization of the colony cells was performed after mass harvesting the colonies of the agar culture. The membrane phenotype studies indicated that colony cells consisted of a mixture of cells bearing either the epitope T4 or T8.

Experiments described above were performed using E⁺ cells incubated with HTLV-I. This cell population was found

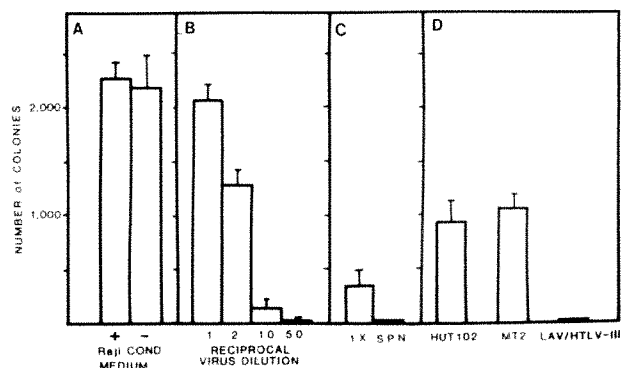


Fig 2. T colony formation, in the absence of IL2: (A) by E⁺ cells infected with HTLV-I (from C91/PL cells), with and without Raji conditioned medium; (B) by E⁺ cells infected with HTLV-I from C91/PL cells, as a dose response relationship with the amount of added virus; (C) by E⁺ cells infected with HTLV-I obtained after low-speed (1x) or high-speed centrifugation (SPN) of C91/PL medium; and (D) by E⁺ cell infected by HTLV-I harvested from two other HTLV-I-transformed cell lines (HUT102 and MT2) or with LAV/HTLV-III, harvested from the medium of H9/HTLV-III cells.

to contain a low percentage of monocytes. To determine whether monocytes were essential to the IL2-independent colony formation, experiments were performed on monocyte-depleted T lymphocytes. It was found that purified T lymphocytes incubated with HTLV-I were still able to generate colonies in the absence of exogenous IL2. However, the addition of 50,000 uninfected monocytes in the agar upper layer was followed by an increase in the number of colonies. The same increase in percentage (around 64%) was observed when uninfected purified T cells were seeded in presence of monocytes (Fig 3).

Finally, it was observed that T colony-forming cells, recruited among monocyte-depleted T4 or T8 lymphocytes, are able to proliferate in the absence of IL2, after incubation with HTLV-I (Fig 3). However, there were twice as many colonies generated with T4 lymphocytes as with those bearing the T8 epitope. Once again, a significant increase in the number of colonies followed the addition of monocytes.

In conclusion, incubation of T colony-forming cells with HTLV-I viral particles promotes a colony-forming ability independent of exogenous IL2, within seven days after plating. Moreover, monocytes, although favoring T colony formation, are not required for the IL2-independent colony generation promoted by HTLV-I.

Binding of HTLV-I particles to T cells is sufficient for colony formation independent of IL2. HTLV-I antigen p19 could not be detected in colony cells, indicating that viral replication was not taking place in these cells during the short time of colony formation. These results lead us to anticipate that T colony ability promoted by HTLV-I infection may not be linked to viral integration and replication,

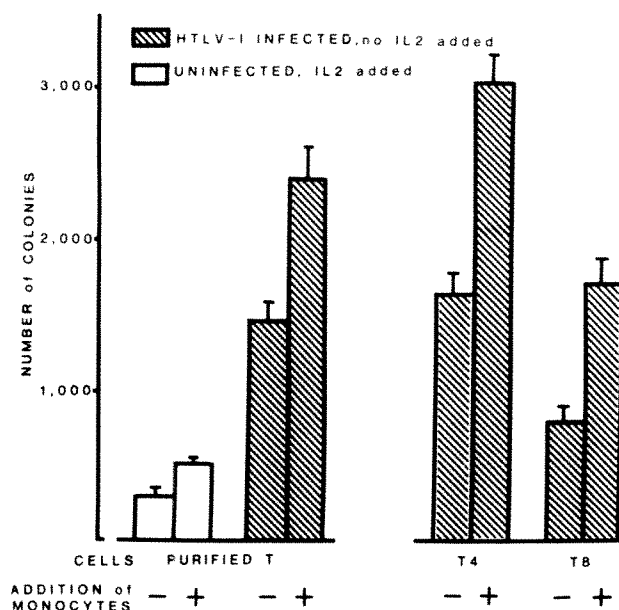


Fig 3. Effect of monocytes on T colony formation by purified (monocyte-depleted) T lymphocytes either uninfected or infected with HTLV-I from C91/PL cells, or by T4 or T8 lymphocytes infected with this virus. Monocytes freshly prepared from the same peripheral blood were added at a density of 50,000 per plate, in the agar upper layer containing 40% of Raji conditioned medium. Colony numbers are expressed as mean $\pm$ SD.

but would follow an early event, such as the binding of HTLV-I virions to the surface of T colony-forming cells.

It is well established that early events in the infection of cells by virus, such as binding and penetration, are mediated by specific viral receptors on the surface of target cells. As assayed by flow cytometry, viral receptors of HTLV-I are more numerous on human T lymphocytes than on B lymphocytes.²⁴ To verify that the induction of T colony formation by HTLV-I particles could be mediated by binding of the virus to receptors on T colony-forming cells, viral particles were heat-inactivated by incubating them at 56 °C for one hour. After this treatment, which inhibits their replicative ability, viral particles were mixed with T cells. Under these conditions, the number and the size of colonies obtained after incubation with heat-treated virus were found to be the same as that observed with heat-untreated virus (Table 2). So, abolishing the replicative potential of the virus did not impair IL2-independent T colony proliferation.

Experiments were then performed to prevent the binding of viral particles to T cells. HTLV-I virions were incubated with sera from two ATL patients. The sera contained specific antibodies against HTLV-I (as determined by enzyme-linked immunosorbent assay [ELISA] and competition assays), and probably reacted with envelope and core polypeptides of the virus, as demonstrated by previous studies.^{25,26} As shown in Table 2, colony formation in the absence of IL2 was inhibited in a dilution-dependent manner by serum A. A significant inhibition was observed in serum B only when it was used undiluted. No such inhibition was observed with an HTLV-I seronegative serum from one individual living in the same endemic area. These results suggest that antibodies present in the sera from ATL patients mask viral antigenic determinants on the envelope of the particles and prevent their binding to T colony-forming cells. The results of these experiments indicate that events leading to IL2-independent T colony formation appear to follow the attachment of HTLV-I to T cells.

Table 2. Effect of Heat-Inactivated HTLV-I and Neutralized HTLV-I on the Number of Colonies Formed by E⁺ Cells, in the Absence of IL2

Treatment Prior Infection	Number of Colonies /Plate	Percentage of Inhibition %
None	2212 $\pm$ 138	—
Heat-inactivated	2074 $\pm$ 513	—
Incubated with		
HTLV-I-negative serum, undiluted	2081 $\pm$ 181	—
HTLV-I-positive serum A		
undiluted	31 $\pm$ 19	98.5
diluted 1:2	381 $\pm$ 6	81.7
diluted 1:10	1756 $\pm$ 69	15.7
HTLV-I positive serum B		
undiluted	1356 $\pm$ 169	34.9
diluted 1:2	2143 $\pm$ 56	—
diluted 1:10	2525 $\pm$ 50	—

HTLV-I was heat-inactivated at 56 °C for one hour just before infection of E⁺ cells. Incubation of HTLV-I with serum was carried out at room temperature for one hour just before infection of E⁺ cells. 2 $\times$ 10⁵ HTLV-I-infected cells were seeded per plate. Data expressed as mean $\pm$ SD.

DISCUSSION

Identification and characterization of IL2 has allowed the development of specific and well-controlled assays to study the *in vitro* proliferation and differentiation of T lymphocytes.¹⁻⁴ Among these assays, the T colony assay consisting of culturing either PBL or purified T cells in agar or methylcellulose has permitted the identification of a T cell population endowed with the capacity to form colonies.^{5-8,27} Two prerequisites for a high efficiency of colony formation by T cells are the addition of exogenous IL2 and the presence of accessory cells.

Results reported in this paper indicate that a subset of T colony-forming cells, present in either E⁺ or monocyte-depleted T cells, T4 lymphocytes, or T8 lymphocytes, is able to proliferate in semisolid agar independently of IL2 and accessory cells after contact with HTLV-I. Heat-inactivated virus is also able to induce colony growth independently of IL2. Furthermore, the inhibition of colony formation by T cells with HTLV-I and pretreated with ATL patients' sera containing antibodies to HTLV-I underlines the direct involvement of viral particles in promoting colony formation. As expected, colony formation was not observed with virus-untreated T cells, unless IL2 and accessory cells (or soluble factors produced by these cells) were added to the culture colony medium. These results therefore indicate that inactivated HTLV-I particles or viral glycoproteins are sufficient to promote T colony growth in the absence of any added growth factor. Previous studies^{12,13} on the transforming effects of HTLV-I have shown that T lymphocytes from cord blood or peripheral blood infected with HTLV-I, after cocultivation with irradiated HTLV-I-producing cells, were also able to grow *in vitro* in the absence of IL2. However, this autonomous growth (transforming effect) takes place only several weeks after initiation of the coculture. These observations are different from those reported in this paper, which demonstrate the ability of T colony-forming cells to proliferate independently of extrinsic IL2 within one week after virus addition.

Further experiments with monoclonal antibodies directed against the viral envelope glycoprotein (gp46) are needed to determine whether the viral neutralizing epitope is involved in the IL2-independent proliferation. Moreover, it will be of interest to define at what level on the T cell membrane HTLV-I is acting to trigger cell proliferation. As already mentioned by McGrath and Weissman²⁸ in a study on the relationship of normal immune receptors to retrovirus lymphomagenesis, it is reasonable to consider that HTLV-I is acting at the level of cell surface receptor complexes leading to the activation of the gene program required for T cell proliferation. Several receptors on the surface of T lymphocytes have been implicated in the activation and proliferation of these cells *in vitro*. Among these signals transducing receptors, the CD3-T cell receptor complex and the CD2 E-rosette receptor are mitogenic for T cells upon reaction with specific antibodies recognizing these structures.^{4,29-31} Whether HTLV-I receptors are identical to those surface receptors or are located close to them remains to be investigated.

The finding that T colony formation is independent of

exogenous IL2 suggests that T cell proliferation is induced through one of two mechanisms. The first is related to the direct activation of protein kinase C in the cell membrane, as already described, with PBL treated with the tumor promoter teleocidin.³² The second mechanism is linked to the autocrine secretion of IL2 by T cells in response to HTLV-I. In fact, the lack of T lymphocyte proliferation observed when HTLV-I-treated T cells were seeded at low concentrations might be secondary to their inability to produce sufficient endogenous IL2 (Fig 1).

Spontaneous T cell colony-forming ability has already been described in immature T cells derived from the peripheral blood of patients with T acute lymphoblastic leukemia.³³ Such cells were able to proliferate in methylcellulose in the absence of added growth factors or of mitogenic stimulation, but were partially dependent on factors released by mature T cells. However, it cannot be ruled out that the ability to form T colonies displayed by the leukemic cells may be the consequence of the leukemic process, which interferes with the mechanisms regulating normal proliferation and differentiation.

As underlined above, HTLV-I isolated from the peripheral blood of ATL patients is able to transform T cells *in vitro*. The other known human retrovirus, LAV/HTLV-III, was isolated from the peripheral blood of patients with the acquired immunodeficiency syndrome. This virus replicates exclusively in a subset of T cells, ie, helper/inducer cells with the CD4 phenotype.³⁴⁻³⁶ In contrast to HTLV-I, LAV/HTLV-III is unable to induce T colony formation in the absence of IL2. It is noteworthy that the CD4 epitope, which may be the receptor for this virus,^{34,35} is not involved in T cell activation, since perturbation of the CD4 molecules delivers a negative signal to T lymphocytes.³⁷

The growth of T cells in the absence of exogenous IL2 as reported here underlines an early effect of HTLV-I viral particles on target cells. The fact that such an effect occurs within seven days after the addition of HTLV-I is of paramount importance for the understanding of the mechanisms involved in viral-induced proliferation. Thus, the colony formation induced by HTLV-I in the absence of added IL2 may be considered as a preleukemic event favoring viral integration and viral replication in actively dividing T cells.

ACKNOWLEDGMENT

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Human GM-CSF Primes Neutrophils for Enhanced Oxidative Metabolism in Response to the Major Physiological Chemoattractants

By Richard H. Weisbart, Luz Kwan, David W. Golde, and Judith C. Gasson

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a T cell-derived lymphokine which induces hematopoietic precursor cells to proliferate in vitro and differentiate to neutrophils and macrophages. GM-CSF also inhibits the motility of mature neutrophils (NIF-T activity), and primes neutrophils to enhance oxidative metabolism in response to the bacterial chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP). The present study was designed to determine whether this lymphokine also enhances neutrophil oxidative metabolism in response to the other major physiological chemoattractants which include complement-derived C5a, and the 5-lipoxygenation product of arachidonic acid, leukotriene B₄ (LTB₄). Su-

peroxide anion production was measured as superoxide dismutase-inhibitable cytochrome C reduction. Purified biosynthetic GM-CSF enhanced superoxide anion production by neutrophils in response to f-MLP, C5a desArg, and LTB₄. In contrast to several other factors which prime neutrophils, GM-CSF did not prime for an enhanced oxidative response to phorbol myristate acetate (PMA). These results suggest that GM-CSF may be an endogenous regulator of neutrophil inflammatory responses induced by the major physiological chemoattractants.

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PRODUCTS OF OXIDATIVE metabolism mediate neutrophil (PMN) inflammatory responses and play an important role in host defense.¹ Bacterial-derived *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP), complement-derived C5a, and the 5-lipoxygenation product of arachidonic acid metabolism, leukotriene B₄ (LTB₄) are chemotactic factors for PMN that initiate PMN inflammatory responses by activating oxidative metabolism.²⁻⁵ Recent studies identified a T lymphocyte-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) that primes PMN for enhanced oxidative metabolism in response to f-MLP.⁶⁻⁸ Superoxide anion production was increased three- to fourfold in response to f-MLP by PMN preincubated with purified biosynthetic GM-CSF. Purified GM-CSF alone did not stimulate superoxide anion production. Since C5a and LTB₄ are also chemotactic, it is of considerable interest to determine whether GM-CSF also primes PMN to enhance oxidative metabolism in response to the other major physiological PMN chemoattractants. In addition to GM-CSF, other substances prime PMN, including endotoxin, f-MLP, and a product of B lymphocytes.⁹⁻¹¹ It was also of interest to determine whether the mechanism for priming by GM-CSF was the same as for these other substances.

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MATERIALS AND METHODS

Materials. Phorbol myristate acetate (PMA) (Consolidated Midland Corp, Brewster, NY) was dissolved in absolute ethanol, 0.05 mg/mL, aliquoted and stored at -70 °C until used at a final concentration of 0.05 µg/mL. Purified C5a desArg (Upjohn Diagnostics, Kalamazoo, MI) was used at a final concentration of 8 ng/mL. The purity of C5a desArg was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of radioiodinated C5a desArg which was visualized by radioautography. LTB₄ (Calbiochem, La Jolla, CA) was used at a final concentration of 10⁻⁶ mol/L. f-MLP (Sigma Chemical Co, St. Louis) was dissolved in dimethyl sulfoxide and used at a final concentration of 10⁻⁸ mol/L.

Isolation of human PMN. PMN were isolated from heparinized peripheral blood of healthy subjects by Ficoll-Hypaque gradient centrifugation as previously described.^{12,13} Red blood cells (RBC) were removed by sedimentation in 6% dextran (High Fraction, J.T. Baker Chemical Co, Phillipsburg, NJ). Residual RBC were subjected to hypotonic lysis for 30 seconds. The resulting cell preparations containing 97% PMN were washed and resuspended in Hanks' balanced salt solution (HBSS) (without phenol red) containing 10% fetal calf serum (FCS).

GM-CSF. GM-CSF was purified from serum-free medium conditioned by COS monkey kidney cells transfected with the GM-CSF cDNA clone in the p91023(B) vector as described previously, using gel filtration and reverse phase high performance liquid chromatography (HPLC).^{6,8} Purification of GM-CSF to apparent homogeneity was confirmed by the presence of a single iodinated 22,000-dalton protein band by SDS-PAGE. The protein concentration was determined by amino acid analysis; the specific activity of the purified protein assayed on human bone marrow is approximately 2 × 10⁸ units per mg.¹⁴ Purified GM-CSF was shown to be free of measurable endotoxin by the Limulus amoebocyte lysate assay (Sigma Chemical Co, St. Louis). Purified biosynthetic GM-CSF was used at a concentration of 100 pmol/L to study its effect on PMN oxidative metabolism. Maximal stimulation of colony formation by bone marrow cells and KG-1 cells as well as enhancement of neutrophil oxidative metabolism in response to f-MLP is seen at approximately 100 pmol/L.¹⁵

Assay for superoxide anion. PMN (5 × 10⁶/mL) were suspended in HBSS (without phenol red) containing 10% FCS, and 0.4 mL aliquots containing 2 × 10⁶ cells were incubated for two hours at 37 °C with 0.04 mL of protein diluent (negative control) or GM-CSF. After incubation, PMN were equally divided (1 × 10⁶ PMN/tube) and 0.010 mL of superoxide dismutase was added to one tube at a final concentration of 0.02 mg/mL. C5a or LTB₄ were

Table 1. Effect of Purified Recombinant GM-CSF on PMN Oxidative Metabolism

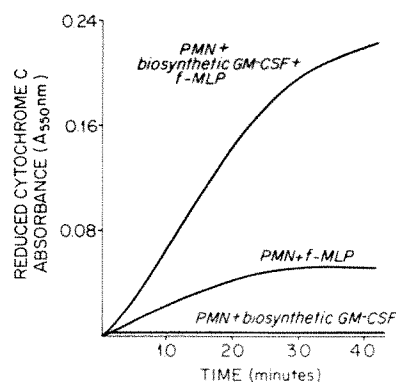
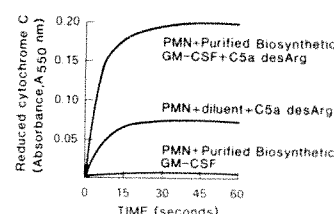
Oxidative Stimulus	Incubation Mixture	No. of Exps	Duration of Experiment (min)	Total Superoxide Anion Produced (nmol)*
f-MLP	diluent	4	4	6.0 ± 0.4
	GM-CSF	4	4	16.0 ± 0.4
C5a desArg	diluent	4	1	1.7 ± 0.1
	GM-CSF	4	1	3.2 ± 0.4
LTB ₄	diluent	4	1	2.4 ± 0.4
	GM-CSF	4	1	4.4 ± 0.4
PMA	diluent	4	25	74.4 ± 3.0
	GM-CSF	4	25	76.8 ± 1.0

*Mean response ± SEM of 10⁶ PMN.

added to each tube, thoroughly mixed, followed by the addition of 1 mL of cytochrome C (1.2 mg/mL in HBSS with 10% FCS). The cell suspensions were transferred to disposable 1-mL cuvettes, and superoxide dismutase-inhibitable cytochrome C reduction was measured spectrophotometrically at 550 nm for one minute at 37 °C with a Varian DMS 90 dual-beam spectrophotometer. Absorbance was recorded continuously with a strip-chart recorder and also at five-second intervals with a computer-assisted program. Superoxide anion production (nmoles) was calculated from reduced cytochrome C using an extinction coefficient of 21,100 mol/L⁻¹ cm⁻¹ and expressed as the mean determination of multiple experiments ± SEM.¹⁶ The effect of GM-CSF on oxidative metabolism of PMN in response to PMA and f-MLP was determined by incubating 2 × 10⁶ PMN with control and test preparations as above, adding 2 mL of cytochrome C, and then equally dividing the cell suspension into separate tubes. Superoxide dismutase was added to one tube, and PMA or f-MLP was added to both tubes. The cells were mixed and immediately transferred to cuvettes where the formation of reduced cytochrome C was monitored for four minutes (f-MLP) or 25 minutes (PMA). The data were recorded and the results calculated as described above.

RESULTS

Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to f-MLP. In preliminary experiments, a dose-response curve was established for f-MLP, and maximum production of superoxide anion occurred at a concentration of 10⁻⁸ mol/L f-MLP. Total superoxide anion produced over four minutes by 10⁶ PMN

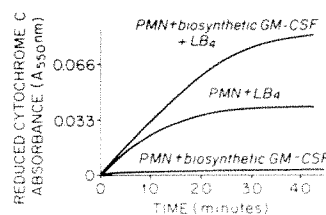
**Fig 1. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to f-MLP.****Fig 2. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to C5a desArg.**

incubated with the protein diluent (negative control) was 6.0 ± 0.4 nmoles in response to 10⁻⁸ mol/L f-MLP (mean response of four experiments). Maximum priming by GM-CSF for oxidative metabolism was shown to occur at two hours.⁷ PMN preincubated for two hours with purified recombinant GM-CSF and then stimulated with f-MLP produced an average of 16.0 ± 0.4 nmoles superoxide anion (mean response of four experiments) (Table 1). A representative example of one experiment is shown in Fig 1. Enhancement of a maximal f-MLP response indicates a total increase in PMN oxidative metabolism and excludes measuring a shift in the f-MLP dose-response curve. The purified preparation of GM-CSF was free of measurable endotoxin (<0.1 ng/mL).

Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to C5a desArg. C5a desArg stimulated the production of 1.7 ± 0.1 nmoles superoxide anion over one minute (mean response of four experiments). PMN preincubated with GM-CSF, however, produced 3.2 ± 0.4 nmoles superoxide anion over the same period of time (mean response of four experiments) (Table 1). A representative experiment is shown in Fig 2.

Effect of purified biosynthetic GM-CSF on superoxide anion production in response to LTB₄. Maximum stimulation of oxidative metabolism of PMN in response to LTB₄ occurred at a concentration of 10⁻⁶ mol/L. At this concentration, LTB₄ stimulated the production of 2.4 ± 0.4 nmoles of superoxide anion by 10⁶ PMN over one minute (mean response of four experiments). Preincubation of PMN with GM-CSF for two hours followed by LTB₄ increased superoxide anion production to 4.4 ± 0.4 nmoles (mean response of four experiments) (Table 1). A representative experiment is shown in Fig 3.

Effect of purified biosynthetic GM-CSF on superoxide anion production in response to PMA. The specificity of PMN priming by GM-CSF was examined by measuring the effect of GM-CSF on PMN oxidative metabolism in

**Fig 3. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to LTB₄.**

response to PMA. In the absence of GM-CSF, 10^6 PMN produced 74.4 ± 3.0 nmoles superoxide anion over 25 minutes which represents the entire course of PMA-induced activation (mean response of four experiments). After preincubating PMN with GM-CSF, the total response was unchanged with 76.8 ± 1.0 nmoles superoxide anion produced (mean response of four experiments) (Table 1). There was also no change in the rate of superoxide anion production. A representative example is shown in Fig 4.

DISCUSSION

PMN are chemoattracted to sites of inflammation where they serve as cytotoxic effector cells. Oxidative metabolism in PMNs is activated by physiological chemoattractants such as bacterial-related f-MLP, serum complement-derived C5a, and the 5-lipoxygenation product of arachidonic acid metabolism, LTB₄, with the production of toxic free radicals of oxygen.²⁻⁵ Products of oxidative metabolism provide one mechanism whereby PMN are cytotoxic.

Lymphokines have been described as endogenous regulators of oxidative metabolism in human monocytes,¹⁷ and colony-stimulating factors have been shown to potentiate granulocyte-mediated cellular cytotoxicity in the murine system.^{18,19} A potential physiological regulator of PMN oxidative metabolism was recently identified as the T cell-derived lymphokine, GM-CSF. GM-CSF, a 22,000-dalton glycoprotein, was shown to enhance the production of superoxide anion by PMN in response to f-MLP.⁷ The present studies were designed to determine whether GM-CSF also regulated PMN oxidative metabolism in response to other physiological chemoattractants.

Our results showed that PMN preincubated with biosynthetic purified GM-CSF enhanced superoxide anion production in response to purified C5a desArg and LTB₄ as well as f-MLP. Each chemoattractant was used at an optimum dose for stimulating superoxide anion production, so enhancement represented an increase in total response and not a shift in the dose-response curve. In contrast to GM-CSF priming for chemoattractants, GM-CSF did not enhance oxidative metabolism in response to PMA. There was no effect on the rate of superoxide anion production or the total amount of superoxide anion produced by PMN during the entire course of PMA activation.

Recently, endotoxin was shown to prime PMN for enhanced oxidative responses to f-MLP.¹⁰ Moreover, the time course for priming was similar to that obtained with GM-CSF. Several observations, however, exclude the possi-

bility that our results were due to the presence of endotoxin. First, HPLC-purified biosynthetic GM-CSF was compared to the HPLC diluent and to the other HPLC fractions that would contain the same contaminants. In addition, endotoxin was not detected by the Limulus amoebocyte assay in HPLC fractions containing neutrophil-activating activity, although the Limulus assay is sensitive enough to detect quantities of endotoxin considerably smaller than those required to prime PMN for enhanced oxidative metabolism.¹⁰ Finally, endotoxin and GM-CSF are different with respect to priming PMN in response to PMA. Endotoxin primes PMN for enhanced oxidative metabolism in response to PMA, whereas GM-CSF does not.

Lipoxygenase products also enhance neutrophil oxidative metabolism in response to f-MLP, but their effect of PMA responses has not been examined.²⁰ It will be of interest to compare the mechanism of action of these products with GM-CSF since these products could be responsible for the GM-CSF effect.

Other substances have also been shown to prime PMN for enhanced oxidative metabolism. In addition to endotoxin, priming has been shown to occur in response to subthreshold concentrations of f-MLP.^{9,21} Moreover, priming occurs in response to a factor present in the supernatant of a B lymphoblast cell line.¹¹ In contrast to GM-CSF, however, f-MLP, the B cell-derived factor, and endotoxin all prime PMN for enhanced oxidative metabolism in response to PMA.^{9,11,22,23} This finding suggests multiple mechanisms for priming with some specificity in the mechanism for PMN priming by GM-CSF. Although the mechanism for PMN priming by GM-CSF is unknown, a variety of secretory stimuli have been shown to increase f-MLP receptor availability by mobilizing an intracellular pool of f-MLP receptors.¹⁶ Moreover, recent studies show that short (15-minute) incubations of GM-CSF with neutrophils resulted in increased expression of high-affinity f-MLP receptors corresponding with enhanced chemotaxis in response to f-MLP, whereas more prolonged incubation of neutrophils with GM-CSF (two hours) resulted in the development of low-affinity receptors corresponding with enhanced oxidative metabolism in response to f-MLP.²⁴

PMN oxidative metabolism provides a mechanism for host defense through the production of potent derivatives of oxygen with microbicidal and tumoricidal activity. PMN oxidative metabolism requires precise regulation and control, as products of oxidative metabolism may induce tissue injury as well. GM-CSF may be an endogenous regulator of PMN oxidative metabolism and contribute to host defense-related reactions. In addition, GM-CSF could play a role in the pathophysiology of tissue injury associated with excessive inflammation as occurs in autoimmune diseases. GM-CSF regulation of PMN oxidative metabolism in response to different physiological chemoattractants may provide a common basis for understanding diverse biological functions of GM-CSF.

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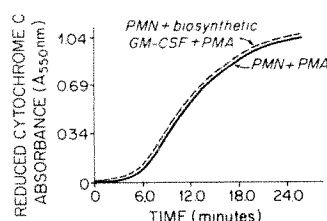


Fig 4. Effect of purified biosynthetic GM-CSF on superoxide anion production by PMN in response to PMA.

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P25713

Activation of Plasminogen by Single-Chain Urokinase or by Two-Chain Urokinase—A Demonstration That Single-Chain Urokinase Has a Low Catalytic Activity (Pro-Urokinase)

By Ralph Pannell and Victor Gurewich

Single-chain urokinase (SC-UK) has an intrinsic amidolytic activity, as measured with synthetic substrate (Kabi S-2444; pyro-Glu-Gly-Arg-pNitroanilide), which was found to be 0.1% to 0.2% that of its plasmin-activated derivative, two-chain UK (TC-UK). A study of the reaction of SC-UK with plasminogen is complicated by the effect of the reaction product, plasmin, on both reactants. The resultant generation of TC-UK and Lys-plasminogen produces secondary reactions which greatly augment plasminogen activation. To confine enzymatic activity to the primary reaction, after pretreatment to eliminate trace TC-UK contaminants, SC-UK was incubated with Glu- or Lys-plasminogen in the presence of aprotinin (500 KIU/mL) to inhibit generated plasmin and dansyl-glutamyl-glycyl-argi-

nyl-chloromethylketone (20 μ mol/L), which irreversibly inhibited TC-UK but not SC-UK. Analysis by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a plasminogen-activating activity for SC-UK that was ~0.4% that of TC-UK. Both SC-UK and TC-UK preferentially activated Lys-plasminogen over Glu-plasminogen. Similarly, Glu-plasminogen activation was augmented by lysine or soluble fibrin. The ratio of the reaction rates of SC-UK and TC-UK were comparable for Glu- and Lys-plasminogen. It is concluded that there is a major difference in the catalytic activities of SC-UK and TC-UK against plasminogen that is comparable to that against synthetic substrate.

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SINGLE-CHAIN, high-mol-wt urokinase (SC-UK) was originally discovered in urine¹⁻³ and has been subsequently purified from the secretions of certain cells in culture^{4,5} and from blood plasma.⁶ This protein has been shown to be a natural precursor of two-chain, high-mol-wt urokinase (TC-UK) and to possess properties characteristic of a proenzyme or zymogen and has thus been referred to as pro-UK.^{4,7-9}

The relationship of SC-UK to TC-UK has obvious analogies within the single-chain:two-chain/zymogen:enzyme paradigm, which is well demonstrated within the serine protease class. Yet in the reaction of SC-UK with its physiological substrate, ie, Glu-plasminogen, its reactivity has been reported to be comparable to that of TC-UK,¹⁰ whereby it was concluded that the name pro-UK is inappropriate and a change in nomenclature to "single chain UK type plasminogen activator (scu-PA)" was recommended.¹¹ The analysis of the activity of SC-UK with plasminogen is complicated by the susceptibility of both reactants to enzymatic alteration by the primary reaction product (ie, Glu-plasmin). These side reactions, resulting in formation of TC-UK and Lys-plasminogen, greatly potentiate plasmin generation and interfere with the analysis of the primary reaction.

To dissect these reactions and to identify the component that is uniquely relevant to SC-UK, we exploited the wide differential between SC-UK and TC-UK in their susceptibilities to UK-reactive active site inhibitors such as diisopropylfluorophosphate (DFP)⁷ and dansyl-Glu-Gly-Arg-chloromethylketone (dns-GGA-ck) (vide infra). The plasmin-mediated side reactions were quenched by a competitive inhibitor, aprotinin, precluding the use of an enzymatic assay. We therefore used electrophoretic analysis of the reaction products. Although this method is not amenable to quantitation sufficient to allow derivation of kinetic parameters, it offers evidence that there is a major differential of net catalysis between SC-UK and TC-UK, which is of a similar quantitative order as has been observed with the synthetic substrate.

MATERIALS AND METHODS

Materials. Single-chain UK (>99% single chain; mol wt 55,000), purified from the culture medium of a human kidney tumor cell line, was provided by Collaborative Research (Lexington, MA). Two-chain UK (mol wt 55,000) was a product of Green Cross (Osaka, Japan). The WHO International Reference Preparation of UK, 66/46 (IRP-UK) was used as the activity standard (obtained from the National Institute for Biological Standards and Control, London). Amidolytic substrate (S-2444) was obtained from Kabi Vitrum (Stockholm, Sweden), aprotinin from FBA Pharmaceuticals (New York), dns-GGA-ck from Calbiochem (San Diego), DFP from Sigma Chemicals (St Louis), and "soluble" fibrin (Desafib) from American Diagnostica (Greenwich, CT).

Glu-plasminogen was purified from DFP-treated fresh plasma essentially by the method of Castellino and Powell.¹² Lys-plasminogen was prepared from purified Glu-plasminogen by the method of Lucas and co-workers.¹³ Plasmin was prepared by activation of plasminogen (2.5 μ mol/L) at pH 8.8 with either an equal volume of a 10% suspension of immobilized UK (Affi-Gel 15; Bio Rad, Richmond, CA) or an equal volume of Streptokinase (10,000 IU/mL) for 30 minutes at 37 °C.

Amidolytic activity of SC-UK or TC-UK. Amidolytic activity was measured with Kabi substrate S-2444 (pyro-Glu-Gly-Arg-pNitroanilide) (0.75 mmol/L) at 37 °C. The reaction buffer was 0.1 mol/L of Tris-HCl (pH 8.8), 0.1 mol/L of NaCl, and 0.1 mg/mL of bovine serum albumin (BSA). One enzyme unit (EU) was defined as

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the amount of enzyme producing a change in absorbance (405 nmol) of 1 absorbance unit each minute. When assayed in this system, the IRP-UK had an activity of 275 EU/100,000 IU.

The activity of SC-UK was expressed in latent international units per milliliter representing the activity after plasmin activation. Activation of SC-UK to TC-UK was accomplished by incubation with an equal volume of plasmin (0.25 μ mol/L) for 30 to 45 minutes (37 °C). The amidolytic assay of the resultant TC-UK was performed in the presence of aprotinin (25 to 50 KIU/mL) to eliminate further plasmin action. The activated form of SC-UK was found to be comparable to TC-UK (~275 EU/mg).

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli¹⁴ using a 10% polyacrylamide gel. Samples were prepared with 10 mmol/L of dithiothreitol and stained with Coomassie-Brilliant Blue G.

DFP treatment. Single-chain UK and plasminogen were treated with 1 mmol/L of DFP in 0.1 mol/L of sodium phosphate (pH 7.2), and 0.1 mol/L of NaCl. Incubation was carried out on ice for 18 hours. Under these conditions, TC-UK was totally inactivated, but full recovery of the latent activity of SC-UK was obtained when assayed after decay of the DFP.⁷ For preparative use, the treated product was dialyzed against 10 mmol/L of sodium acetate (pH 4.8) and 0.15 mol/L of NaCl and stored frozen.

Stability of SC-UK to dns-GGA-ck. At a concentration of 20 μ mol/L of dns-GGA-ck, complete and irreversible inhibition of TC-UK was obtained within 30 minutes at 37 °C or at room temperature. To test the stability of SC-UK to this inhibitor, SC-UK (0.1 mg/mL) was incubated with 20 μ mol/L of dns-GGA-ck for 2 hours at 37 °C in 0.2 mol/L of HEPES (pH 7.4), 0.3 mol/L of NaCl, 0.1% Triton X-100 (Sigma, St Louis). The solution was then diluted to .025 mg/mL of SC-UK with a buffer of 0.2 mol/L of Tris (pH 8.8), 0.3 mol/L NaCl and 0.2 mg/mL of BSA, and was allowed to incubate further at room temperature for 16 hours. Under the latter condition, dns-GGA-ck has been found to decay completely. The treated SC-UK and a control sample treated without dns-GGA-ck were then activated with plasmin and assayed with S-2444.

Evaluation of the influence of hydrolyzed dns-GGA-ck on the intrinsic activity of SC-UK. To test the effect of the tripeptide of dns-GGA-ck as a potential competitive inhibitor independent of noncompetitive inhibition by the reactive species, a hydrolysis-inactivated sample of the inhibitor was prepared and assayed. Dns-GGA-ck was inactivated by hydrolysis by dilution in 0.1 mol/L of Tris (pH 8.8), 0.3 mol/L of NaCl, and 0.2 mg/mL of BSA, and storage at room temperature for 3 days. The resulting product was assayed as a potential competitive inhibitor against both TC-UK and the inherent activity of SC-UK, using the amidolytic assay. The substrate tripeptide is identical with the tripeptide of dns-GGA-ck. The substrate was at an assay concentration of 0.75 mmol/L; the hydrolyzed inhibitor was tested up to 50 μ mol/L. No significant inhibition was observed with either SC-UK or TC-UK.

Plasminogen degradation by SC-UK or TC-UK. Glu-plasminogen or Lys-plasminogen (0.23 mg/mL) and either SC-UK (5,000, 10,000, or 20,000 IU/mL) or TC-UK (5, 10, or 20 IU/mL) were incubated (37 °C) in a reaction medium comprising 0.2 mol/L of HEPES-HCl (pH 7.4), 0.3 mol/L of NaCl, 0.01% Triton X-100, and aprotinin (500 KIU/mL). SC-UK was pretreated with the dns-GGA-ck (80 μ mol/L at room temperature for 30 minutes in the reaction buffer) prior to dilution into the assay to eliminate residual TC-UK activity, and dns-GGA-ck (20 μ mol/L) was present in the reaction mixture for SC-UK (but not for TC-UK). At 1 and 2 hours, reactions were stopped by dilution of aliquots with SDS sample buffer (reducing).

To test the effect of occupancy of the lysine binding sites of Glu-plasminogen on its susceptibility to SC-UK, the reaction was

run as described above for 2 hours but with the addition of either 0.25 mg/mL of soluble fibrin or 25 mmol/L of lysine. A concentration of 5,000 IU/mL SC-UK or 25 IU/mL of TC-UK was used.

RESULTS

Effect of inhibitors on the amidolytic activities of SC-UK and TC-UK. Preparations of SC-UK were found to have a low but variable specific activity against the synthetic substrate S-2444 as expressed by the ratio of activities before and after plasmin treatment. The variability in this measurement could be attributed to even very small contamination with TC-UK. When preparations were pretreated with DFP (1 mmol/L), sufficient to abolish the activity of the contaminant, a more consistent result was obtained, which showed that SC-UK had an inhibitor resistant activity corresponding to 0.1% to 0.2% of its latent activity as previously observed.¹⁵

A similar result was obtained with the more specific inhibitor dns-GGA-ck. Treatment with 20 μ mol/L of dns-GGA-ck completely inactivated TC-UK, whereas after provision for decay of the inhibitor by hydrolysis (described in the Methods section), the plasmin activatable activity of SC-UK was at least 97% recoverable.

Effects of inhibitors on the reaction of SC-UK with plasminogen. In mixtures of SC-UK with plasminogen (whether Glu or Lys), there was rapid activation of the plasminogen and conversion of SC-UK to the two chains of TC-UK, indicating that TC-UK was being generated and possibly was dominating the reaction. When aprotinin (100 KIU/mL) and dns-GGA-ck (20 μ mol/L) were included in the reaction mixture, the activation of plasminogen was greatly decreased even though electrophoretic analysis revealed that significant traces of SC-UK were still being converted to TC-UK. When the concentration of aprotinin was increased to 500 KIU/mL, however, the secondary reaction of SC-UK activation appeared to be prevented.

Comparison of the activation of Glu-plasminogen by SC-UK and TC-UK. Under these conditions limiting the secondary reactions, the activity of SC-UK was quantitatively compared with that of TC-UK. With both forms of UK, there was a concentration- and time-dependent generation of the heavy (mol wt 67,000) and light (mol wt 25,000) chains of plasmin (Figs 1A and B). Densitometric scanning of the gels showed that the plasmin produced by 10,000 IU/mL of SC-UK in 1 hour was approximately equal to that which occurred with 20 IU/mL of UK in 2 hours. Thus, a ratio of ~250 was calculated, ie, SC-UK was 0.4% as active as TC-UK against plasminogen. This ratio was comparable to its activity against synthetic substrate (0.1% to 0.2%).

Comparison of the activations of Lys-plasminogen and Glu-plasminogen. When Lys-plasminogen was substituted for Glu-plasminogen in the above reaction mixtures, the rate of conversion by both activators was increased, as shown in Figs 2A and B, representing 1 and 2 hours of incubation, respectively. A similar differential between the activities of TC-UK and SC-UK was seen with Lys- as with Glu-plasminogen (5,000 IU/mL of SC-UK $\times$ 1 hour was comparable to 10 IU/mL of TC-UK $\times$ 2 hours, or a ratio of ~250).

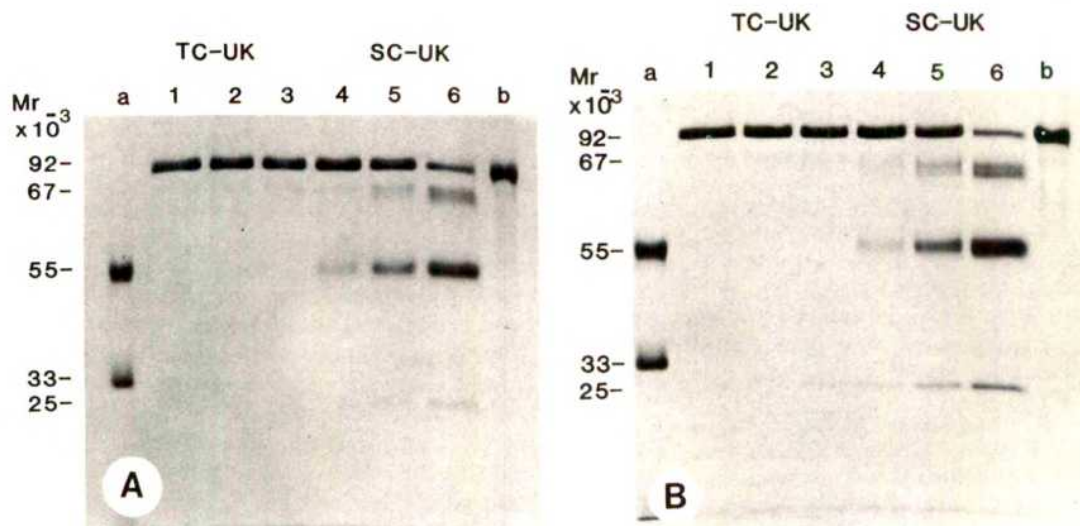


Fig 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of mixtures of Glu-plasminogen, aprotinin (500 KIU/mL), dns-GGA-cK, and activators incubated (37 °C) for 1 hour. Left: (a) two-chain urokinase (TC-UK) plus single-chain urokinase (SC-UK) marker; TC-UK at (1) 5, (2) 10, and (3) 20 IU/mL. Right: SC-UK at (4) 5,000, (5) 10,000, and (6) 20,000 IU/mL; (b) Glu-plasminogen marker. The heavy and light chains of Glu-plasmin appear at mol wts 67,000 and 25,000, respectively. (B) SDS-PAGE of the mixtures shown in A after 2-hour incubation.

Effect of lysine and of soluble fibrin on Glu-plasminogen activation. When lysine or soluble fibrin was used as a modifier of Glu-plasminogen, the observed increased susceptibility of Lys-plasminogen to activation was found to extend to this conformationally modified form. Quantitation by densitometric scanning of the gel shown in Fig 3 showed that whereas 5,000 IU/mL of SC-UK activated <15% of Glu-plasminogen in 2 hours, the inclusion of 25 mmol/L of lysine enhanced activation to ~45%. Soluble fibrin at 0.25 mg/mL gave a significant but smaller enhancement to ~30% conversion.

DISCUSSION

When plasminogen activation was measured under conditions designed to isolate the reaction inherent to SC-UK, a consistent magnitude of discrepancy was observed between SC-UK and TC-UK in their abilities to activate either Glu-plasminogen or Lys-plasminogen. The activity of SC-UK was ~0.4% that of TC-UK, a differential comparable to their respective rates of hydrolysis of synthetic substrate S-2444. These findings are compatible with other zymogenic characteristics of SC-UK, such as its relative resistance to

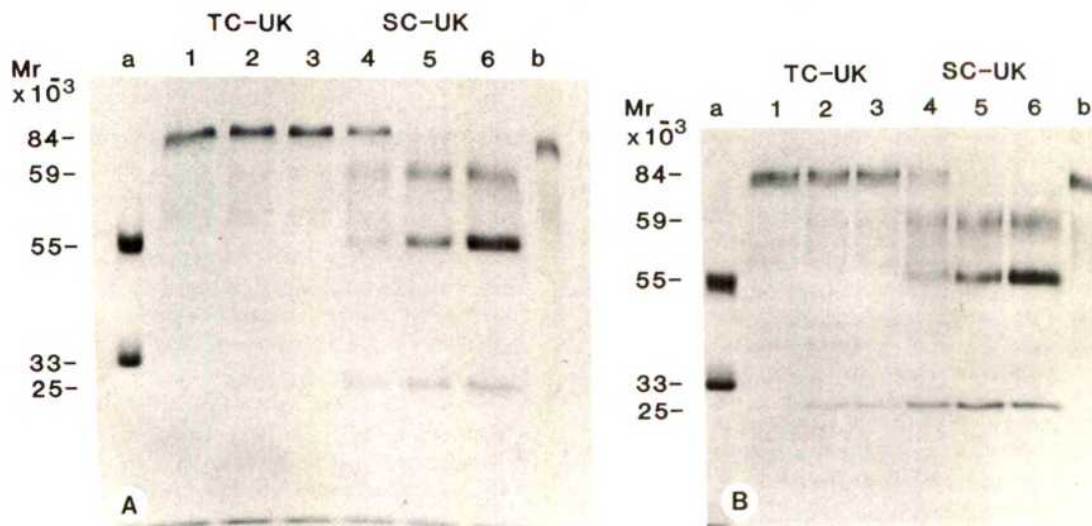


Fig 2. (A) Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mixtures of Lys-plasminogen, aprotinin (500 KIU/mL), dns-GGA-cK and two-chain urokinase (TC-UK) at (1) 5, (2) 10, and (3) 20 IU/mL and single-chain urokinase (SC-UK) at (4) 5,000, (5) 10,000, and (6) 20,000 IU/mL incubated (37 °C) for 1 hour; (a) TC-UK plus SC-UK marker; (b) Lys-plasminogen marker. The heavy and light chains of Lys-plasmin appear at mol wts 59,000 and 25,000, respectively. (B) SDS-PAGE of mixtures shown in A after 2-hour incubation.

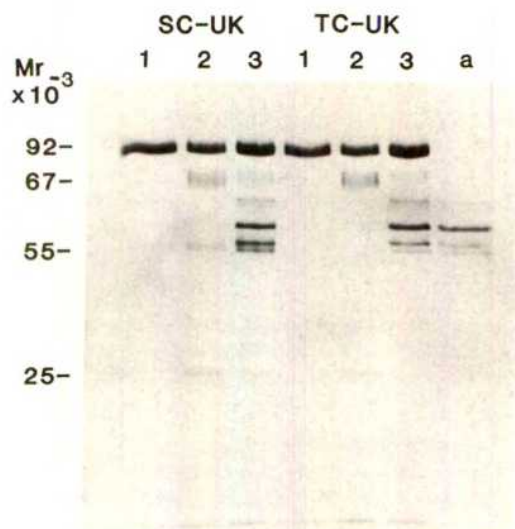


Fig 3. Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of mixtures of Glu-plasminogen incubated (37 °C) for 2 hours with single-chain urokinase (SC-UK) (5,000 IU/mL) (1) alone, (2) plus lysine, and (3) plus soluble fibrin; or two-chain urokinase (TC-UK) (25 IU/mL) (1) alone, (2) plus lysine, and (3) plus soluble fibrin; (a) soluble fibrin marker. In lanes 2 and 3, the appearance of the heavy and light chains of plasmin are seen at mol wts 67,000 and 25,000, respectively.

inhibitors like DFP or dns-GGA-ck, and its stability in plasma.⁸

The observance of such a differential between the reactivities of TC-UK and of SC-UK with the physiological substrate, Glu-plasminogen, is at variance, however, with the report of Collen and colleagues¹⁰ in which the activity of SC-UK was stated to have a catalytic efficiency comparable to that of TC-UK. The discrepancy must be evaluated in terms of the methods of analysis used and particularly the measures taken to eliminate side reactions, principally the generation of TC-UK. These authors used the competitive influence of the synthetic plasmin substrate itself (S2251 at 1 mmol/L) to inhibit the action of plasmin on SC-UK at a concentration that is four times the K_m but that occupies plasmin only to the extent of 80% of its V_{max} (calculated from Michaelis-Menten equation). Therefore, plasmin inhibition may not have been complete. Moreover, the product of this substrate, bearing a carboxy-terminal lysine residue, has the potential to modify Glu-plasminogen by occupying the lysine binding site which promotes its activatability either by UK¹⁶

or by pro-UK (Fig 3). Ultimately, the confirmation of one view or the other must await the application of additional experimental approaches to the problem of isolating the primary reaction.

Evidence for a low inherent activity of SC-UK has also been observed in other systems. Preparations free of TC-UK contamination induce fibrinolysis on a standard fibrin plate and in a model plasma clot lysis system.^{7,8} We have proposed that this inherent activity of SC-UK may constitute a trigger that leads to plasmin generation on the clot surface primarily by way of the positive feedback of the secondary reaction of TC-UK production.^{7,17} The side reaction that is triggered by plasmin activation of SC-UK, posing methodologic complications, probably serves as a useful physiologic amplifier, greatly increasing the rate of localized plasmin generation.

Although the intrinsic activity of the SC-UK is not incompatible with its proenzyme designation,¹⁸ the magnitude of this activity is relatively large. Therefore, SC-UK may be seen as occurring somewhere along a spectrum defined by an absolutely inactive zymogen at one extreme and the fully activated enzymatic form, here represented by TC-UK, at the other extreme. The appropriate place for SC-UK along this spectrum has been defined here only in qualitative terms. Quantitative appraisal of that position has been thwarted by the methodologic problems of measuring the kinetics of the primary reaction of SC-UK and Glu-plasminogen in functional isolation.

With Lys-plasminogen as substrate, plasmin generation was significantly more rapid with either activator, although the ratio of their reaction rates remained comparable. Similarly, a potentiation of plasmin generation occurred when lysine or soluble fibrin was added to Glu-plasminogen reaction mixtures. This effect of lysine or soluble fibrin is related to the conformational change induced in Glu-plasminogen by occupancy of the fibrin/lysine binding site(s).¹⁹ We recently proposed that the preferential activation of fibrin-bound plasminogen is an important element of the mechanism responsible for the fibrin specificity of SC-UK.¹⁵ A preferential activation of Lys-plasminogen over Glu-plasminogen has previously been reported for TC-UK.¹⁹

In conclusion, under conditions in which the primary reaction inherent to SC-UK was isolated and compared with that of TC-UK, a major difference was consistently observed between their respective activities, whether as hydrolysis of the synthetic substrate S-2444, reaction with inhibitors such as DFP or dns-GGA-ck, or proteolytic activation of either Glu-plasminogen or Lys-plasminogen.

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Topology and Order of Formation of Interchain Disulfide Bonds in von Willebrand Factor

By Denisa D. Wagner, Sarah O. Lawrence, Betsy M. Ohlsson-Wilhelm, Philip J. Fay, and Victor J. Marder

Interchain disulfide bonds between the subunits in von Willebrand factor (vWf) dimers and in vWf multimers have been studied using some unique features of the cultured human umbilical vein endothelial cell system. Ammonium chloride inhibition of multimerization of vWf allowed selective examination of vWf dimeric molecules, and monoclonal antibody against the vWf propolypeptide was used to separate pro-vWf dimers from mature dimers. After cleavage of dimers and multimers with *Staphylococcus aureus* V-8 protease, the location of interchain disulfide bonds in amino (N)-terminal or carboxyl (C)-terminal frag-

ments was determined by gel electrophoresis under reduced and nonreduced conditions. The first interchain disulfide bonds formed during dimerization are in the C-terminal region of the subunits, whereas interdimer disulfide bonds are located in the N-terminal portion. These data confirm recent electron microscopic projections of disulfide bond locations and provide support to the hypothetical role of the propolypeptide in the multimerization process.

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THE LARGEST von Willebrand factor (vWf) multimers are the most active in in vitro platelet aggregation and binding assays, and their absence in vivo results in type IIA von Willebrand disease (vWd).¹ Therefore, the formation of the interchain disulfide bonds is a crucial aspect of vWf biosynthesis. Studies using cultured endothelial cells indicate that dimers of pro-vWf subunits are formed in the endoplasmic reticulum, and only after these are transported to the Golgi apparatus does interdimer disulfide bond formation begin.² This latter process is pH sensitive because it is inhibited in the presence of a weak base such as ammonium chloride.³ Simultaneous with multimerization, the vWf propolypeptide (von Willebrand antigen II),⁴ located at the N-terminal end of the vWf subunit,⁵⁻⁷ is proteolytically removed,^{8,9} and vWf is secreted into the culture medium or stored in the Weibel-Palade bodies.¹⁰

Many multimeric sizes of vWf are found in plasma.¹ The smallest circulating species is a dimer^{11,12} that could correspond to the dimeric molecule formed in the endoplasmic reticulum. Limited proteolysis of plasma vWf with *Staphylococcus aureus* V-8 protease produces a single cleavage in the vWf subunit, yielding two large homodimers,^{13,14} indicating that multimerization occurs in a head-to-head and tail-to-tail fashion. Electron microscopic observations confirm this type of arrangement and in addition reveal the vWf multimers to be elongated molecules with the interchain disulfide bonds located near the N-terminals and C-terminals of the subunits.^{12,15} In this study, we examine which of these interchain disulfide bonds is formed first (ie, in the endoplasmic reticulum) during dimerization of vWf and compare our result to a preliminary electron microscopic observation which assigns the interchain link of the smallest circulating species as being between the C-terminal regions of the vWf subunits.¹⁶

MATERIALS AND METHODS

Cells and culture conditions. Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described previously^{10,17}; only primary cultures were used. Cells were cultured in McCoy's 5A medium (Flow Laboratories, Inc, McLean, VA) containing 20% fetal bovine serum (FBS). Ammonium chloride (Fischer Scientific, Rochester, NY) was dissolved in culture medium. For metabolic labeling, cells were grown for 3 days in the presence of [³⁵S]methionine (25 μ Ci/mL, 1148 Ci/mmol) (New England Nuclear, Billerica, MA).

Antibodies. Anti-vWf antiserum was purchased from Calbiochem-Behring (San Diego). Antiserum against human pro-vWf was prepared by immunization of New Zealand albino rabbits with purified von Willebrand antigen II (vW AgII) and vWf. Antiserum was rendered monospecific for pro-vWf by filtration through Sepharose CL-4B coupled with plasma from a patient with severe vWd. This polyclonal antiserum recognizes both the vWf propolypeptide (vW AgII) and the mature vWf multimers. Monoclonal antibodies against the 100-kd propolypeptide of vWf¹⁸ were prepared as follows: Spleen cells from female BALB/c mice hyperimmunized with the propolypeptide were fused to the myeloma cell line P3X63Ag8.653¹⁸ at a ratio of 3:1, using standard techniques with HAT selection.¹⁹ Supernatants from each resulting clone were tested by enzyme-linked immunosorbent assay (ELISA) against the propolypeptide, mature vWf, and fibronectin. Antibody-producing clones with preferential activity against the 100-kd propolypeptide of vWf were expanded and recloned until clonal homogeneity was achieved (two to three subclonings). These were then frozen and stored. The specificity of antibody produced by such clones was confirmed by Western blotting.²⁰

Purification of vWf. vWf was immunopurified as described previously.² For purification of molecules containing the propolypeptide, 300 μ L of supernatant containing monoclonal antibody was used for each sample.

***S aureus* V-8 cleavage.** Purified vWf in electrophoresis sample buffer containing 2% sodium dodecyl sulfate (SDS)²¹ was diluted twofold in water containing *S aureus* V-8 protease so that the final concentration of the enzyme was 10 μ g/mL. After 10-minute digestion at room temperature, the reaction was stopped by adding 1/10 vol of 20% SDS and boiling. Samples were immediately prepared for electrophoresis with or without dithiothreitol.

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Electrophoresis gels. Agarose horizontal slab gels were prepared using a solution of 2% agarose, 0.1% SDS in 0.05 mol/L of phosphate buffer at pH 7.0, and running buffer was 0.1 mol/L of phosphate buffer, pH 7.0, with 0.1% SDS. SDS polyacrylamide gels were prepared as described by Laemmli.²¹ For two-dimensional gel electrophoresis, protein was first run nonreduced on a 5% polyacrylamide gel. The strip was cut out and incubated with constant shaking for 20 minutes at room temperature in running buffer²¹ that contained 50 mmol/L of dithiothreitol. The strip was then transferred to the top of an identical 5% SDS polyacrylamide gel and overlaid with 0.5% agarose and 0.1 mol/L of dithiothreitol in running buffer before electrophoresis. Western blotting analysis was performed as previously described.²² The primary antibody solution consisted of a mixture of three different anti-vWf propolypeptide monoclonal antibody supernatants each diluted 1:25.

RESULTS

Ammonium chloride inhibits interdimer disulfide bond formation and partially blocks cleavage of prosequence.³ Therefore, in its presence, endothelial cells in culture secrete dimeric molecules, some of which contain pro-vWf subunits (Fig 1). *S aureus* V-8 protease cleaves the vWf or pro-vWf subunit in two fragments (refs. 13 and 14 and our preliminary results). As diagrammed schematically in Fig 1, the C-terminal fragments released from the two types of subunit would have to be identical, whereas the N-terminal fragment from the pro-vWf subunit should be larger, because it contains in addition the 100-kD prosequence. To localize the interchain disulfide bond(s) in the dimers to the N-terminal or C-terminal portion of the molecule (options A or B, Fig 1), we generated these V-8 protease fragments and determined which were dimeric in the absence of reduction.

Human umbilical vein endothelial cells were metabolically labeled in the presence of 25 mmol/L of ammonium chloride. Culture medium was collected, and molecules containing the pro-vWf sequence were purified by the use of monoclonal antibody prepared against the propolypeptide. vWf molecules containing mature subunits were then isolated from the supernatant with a polyclonal anti-vWf antiserum. The purified protein was examined nonreduced on a 2% agarose gel (Fig 2A), and both reduced and nonreduced protein was examined on a 5% polyacrylamide gel (Fig 2B). The sample purified with the antipropolypeptide monoclonal antibody contained mostly dimers of pro-vWf subunits and free propolypeptide.⁴ The protein purified with the polyclonal antiserum was composed predominantly of dimers of mature

subunits and did not contain the free propolypeptide (Fig 2). Two major bands were produced by V-8 protease cleavage of the mature vWf and pro-vWf dimers. One band was shared between the two samples (Fig 2B, lanes 2 and 4 reduced, lanes 6 and 8 nonreduced) and therefore represents the C-terminal fragment (CTF). As expected, the other N-terminal cleavage fragment was larger when obtained from the pro-vWf dimer (pro-NTF) than from mature vWf dimers (NTF) (Fig 2b). The identification of the fragments was further confirmed by Western blotting as follows: a mixture of pro-vWf and vWf dimers purified from culture medium of ammonium chloride-treated cells by polyclonal antiserum against pro-vWf were digested by V-8 protease. The starting material and the resulting fragments were electrophoresed on a reduced 6% polyacrylamide gel. Western blots of this gel with a mixture of antipropolypeptide monoclonal antibodies (Fig 3) identified the pro-vWf subunit, the propolypeptide, and the pro-NTF, and demonstrated that the large pro-NTF indeed contains the N-terminal portion of the molecule.

Both pro-NTF and NTF migrated faster on gels nonreduced, indicative of the presence of intrachain disulfide bonds (Fig 2B). On the other hand, the C-terminal fragment migrated significantly more slowly nonreduced, suggesting that it was dimeric. To show this more convincingly, the *S aureus* V-8 proteolytic fragments were examined by two-dimensional gel electrophoresis (Fig 4). In the first dimension, the samples were electrophoresed nonreduced on a 5% polyacrylamide gel; then the lanes were cut out, incubated in the presence of a reducing agent, and placed on top of a second 5% polyacrylamide gel (as shown in Fig 4) and electrophoresed. Only CTF migrated well below the diagonal of the gel, confirming that it was dimeric prior to reduction. It appears therefore that the dimers that are the building blocks of vWf polymers^{2,23} are held together by disulfide bond(s) localized in the C-terminal region of the polypeptide chain (option B, Fig 1).

The major products of *S aureus* V-8 cleavage of plasma vWf are two large distinct dimeric fragments,^{13,14} indicating that both ends of the vWf subunit form interchain disulfide bonds. Because the first link between the subunits of dimers is in the C-terminal region, the interdimer disulfides formed later in the processing of vWF² should involve the other (N-terminal) end of the subunit. If this is so, the amount of the monomeric N-terminal fragment should decrease with an increase in size of vWf multimers submitted to *S aureus*

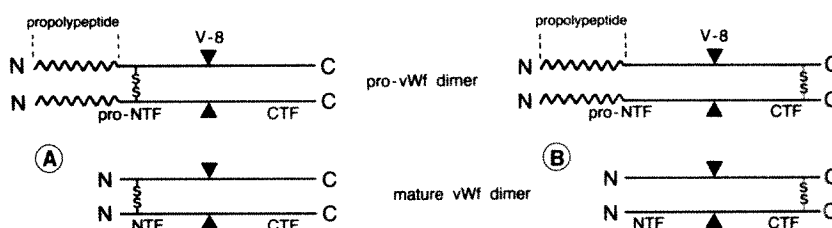


Fig 1. Schematic representation of the location of disulfide bonds in the pro-vWf dimer and mature vWf dimer. The arrowheads indicate the approximate cleavage site for *Staphylococcus aureus* V-8 protease. Following cleavage, V-8 protease produces N-terminal fragments (pro-NTF and NTF) and C-terminal fragments (CTF). If the interchain disulfide bond(s) of the dimers is located at the N-terminal part of the molecule (option A), the pro-NTF and NTF will be dimeric, whereas a location of the disulfide bond(s) at the C-terminal end (option B) will produce dimeric CTF.

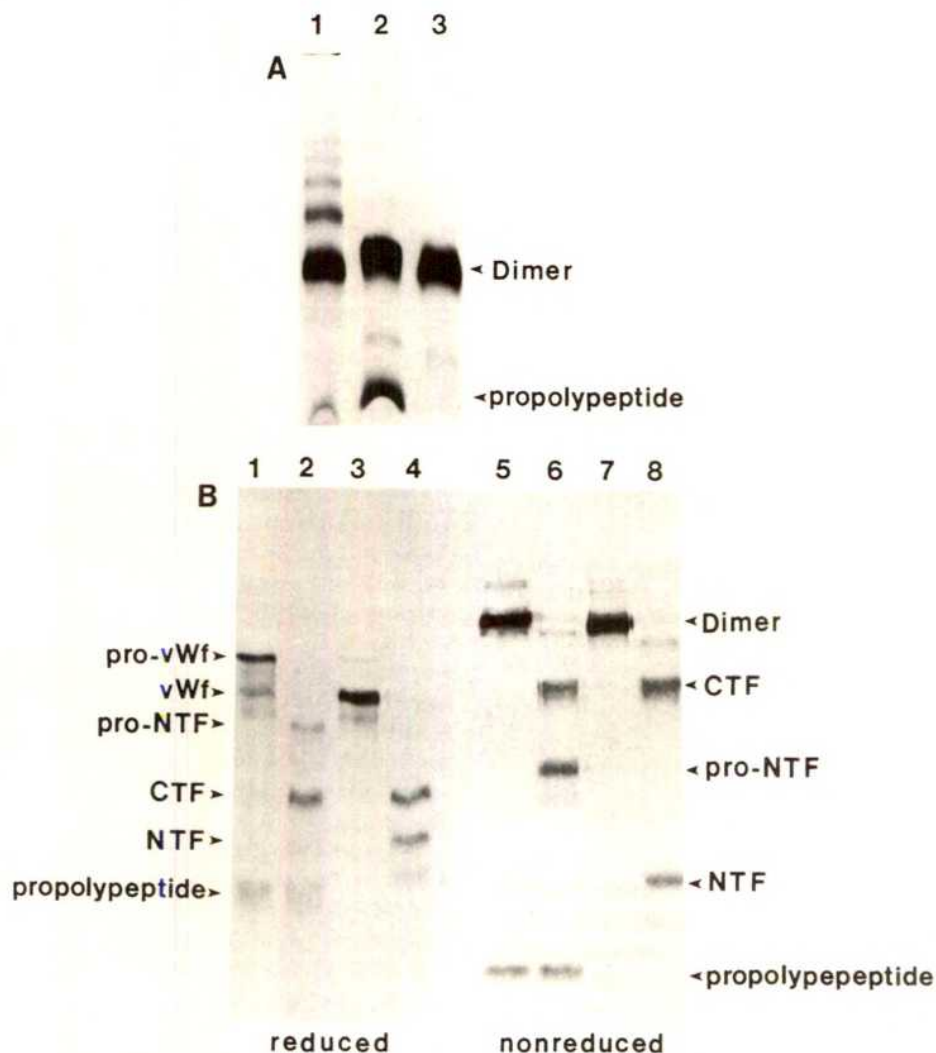


Fig 2. *Staphylococcus aureus* V-8 cleavage of pro-vWf and vWf dimers. (A) The wide range in size of vWf multimers secreted constitutively by metabolically labeled endothelial cell cultures is indicated in lane 1. In the presence of 25 mmol/L of ammonium chloride, only vWf dimers are produced, and these can be separated immunologically (described in text) into pro-vWf dimers (lane 2) and mature vWf dimers (lane 3). Purification of the pro-vWf dimer with specific monoclonal antibody against the prosequence also precipitates free propolypeptide. Autoradiograph of a 2% agarose gel is shown. (B) Autoradiograph of a 5% polyacrylamide gel, the left portion of which (lanes 1 through 4) was obtained with reduced protein. Pro-vWf dimers were examined before V-8 protease cleavage (lanes 1 and 5) and after cleavage (lanes 2 and 6). Mature vWf dimers are similarly shown before protease cleavage (lanes 3 and 7) and after cleavage (lanes 4 and 8). The 100-kd propolypeptide is not cleaved by the V-8 protease. Comparison of migration of the fragments with mol wt markers gives the following mol wts: pro-NTF 200 kd, CTF 145 kd, and NTF 125 kd. Because vWf and pro-vWf migrate anomalously in polyacrylamide gels,⁴ these estimated mol wts are likely incorrect. The minor band which migrates more rapidly than the pro-NTF and NTF fragments is a secondary cleavage product of these fragments. The small amount of mature vWf subunit in the pro-vWf preparation (lane 1) indicates that a minor fraction of the dimers synthesized in the presence of ammonium chloride are heterodimeric, that is, they contain both types of subunits. The small amount of pro-vWf subunit present in the vWf preparation (lane 3) reflects incomplete prior removal of all pro-vWf by the antipropolypeptide monoclonal antibody.

V-8 digestion. vWf purified from endothelial cell's culture medium (using anti-vWf antiserum) is predominantly composed of small vWf multimers²² (Fig 2a), whereas the protein stored in the endothelial cells is highly multimerized.²⁴ In the endothelial cell's endoplasmic reticulum, there is in addition a large pool of pro-vWf dimers that we can separate from the mature vWf with antipropolypeptide monoclonal antibodies. Metabolically labeled endothelial cell lysate was therefore subjected to a two-step purification procedure. The first step removed the prosequence containing molecules that were

discarded, and the second isolated the remaining high-mol-wt (HMW) vWf multimers, using a polyclonal anti-vWf antiserum. These large cellular multimers were subjected to *S. aureus* V-8 digestion in parallel with the predominantly small multimers purified from the culture medium. The starting material and the resulting fragments were analyzed nonreduced on 6% acrylamide gels (Fig 5). Significantly less monomeric N-terminal fragment was generated from the cellular sample (Fig 5, lane 4) than from the secreted vWf (Fig 5, lane 2), indicating that this portion of the subunit

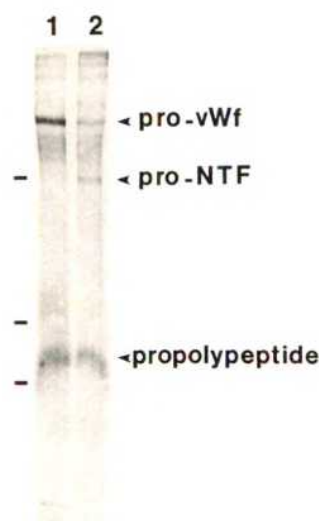


Fig 3. Western blot of *Staphylococcus aureus* V-8 cleavage products with antipropolypeptide monoclonal antibodies. Culture medium from metabolically labeled cells in the presence of 25 mmol/L of ammonium chloride was purified with polyclonal anti-pro-vWf antiserum and electrophoresed reduced on a 6% polyacrylamide gel before (lane 1) and after (lane 2) digestion with V-8 protease. The Western blot of this gel was done with a mixture of three monoclonal antibodies directed against the propolypeptide. Only the pro-NTF, not the CTF and NTF, reacted positively with the antibodies. Indications at left show migration of markers: myosin, 200 kd; β -galactosidase, 130 kd; phosphorylase b, 92.5 kd.

became highly involved in interchain disulfide bonding. Two-dimensional gel electrophoresis of the digested samples (not shown) revealed that the slowly migrating doublet contains both the dimeric C-terminal fragment and the dimeric N-terminal fragment. Because the *S aureus* V-8 cleaves the vWf subunit close to its center, the dimers of the resulting fragments appear similar in size in our gel system. The doublet can be better resolved on low-percentage acrylamide gels in which the CTF dimer migrates slower than the

NTF dimer (not shown). The very small amount of monomeric N-terminal fragment (seen only after long autoradiographic exposures) produced from HMW vWf multimers confirms that this is the part of the subunit involved in the polymerization of the vWf dimers.

DISCUSSION

Although most of the amino acid sequence of the mature vWf subunit is known from molecular cloning studies⁵⁻⁷ and protein sequencing,²⁵ the exact number and location of the interchain disulfide bonds in the vWf multimers remains to be elucidated. To localize the interchain disulfide bonds in vWf multimers, the structure of the protein was first simplified to dimers. This was accomplished by culturing human umbilical vein endothelial cells in the presence of a weak base (ammonium chloride) that inhibits the intracellular multimerization of dimers.³ The resulting dimeric molecules were separated into two groups, namely, those containing pro-vWf subunits and those built of mature subunits. For this purpose, we used monoclonal antibodies prepared against the vWf propolypeptide. The two sets of dimers were submitted to cleavage with *S aureus* V-8 protease, and the resulting fragments were compared reduced and nonreduced and by two-dimensional gel electrophoresis. A shared C-terminal fragment was shown to be dimeric (Figs 2 and 4). Therefore, the first interchain disulfide bonds formed in the biosynthesis of vWf during dimerization are in this C-terminal region of the subunit.

We cannot reconcile why our results showed a C-terminal fragment that was larger than the N-terminal fragment whereas other researchers have reported opposite results following V-8 digestion of mature vWf.¹³ It is possible that the presence of SDS in our samples uncovered a different primary cleavage site for the protease, also localized in the center of the subunit, or that the fragments have different electrophoretic properties in our gel system. The sum of the apparent mol wts of the two fragments, ie, 145 plus 125 kd, corresponds to the size of vWf subunit (270 kd) determined

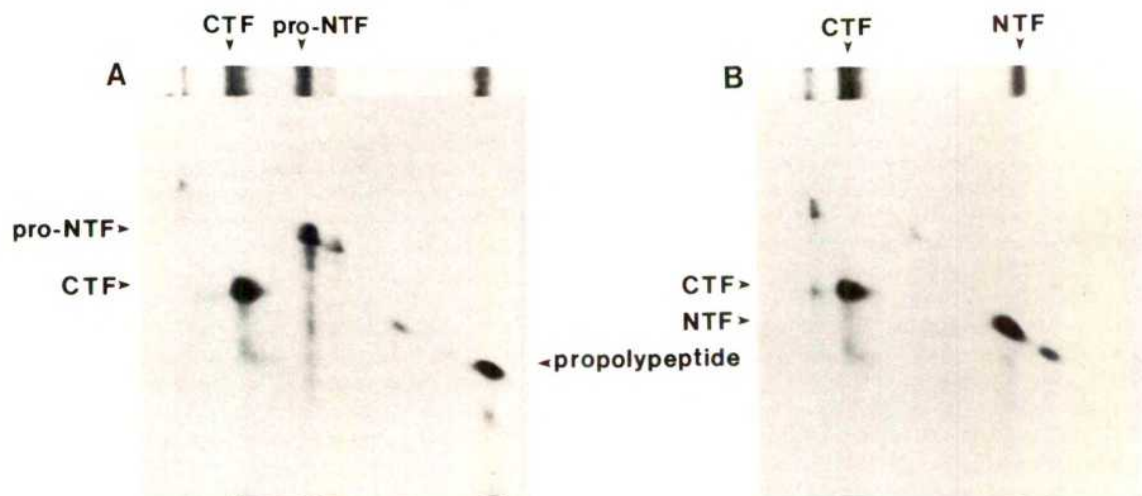


Fig 4. Two-dimensional gel electrophoresis of *Staphylococcus aureus* V-8 cleavage products (autoradiographs of 5% polyacrylamide gels). Samples were analyzed nonreduced in the first dimension as shown (left to right) and reduced in the second (top to bottom). Fragments were obtained from (A) pro-vWf dimers and (B) mature vWf dimers.

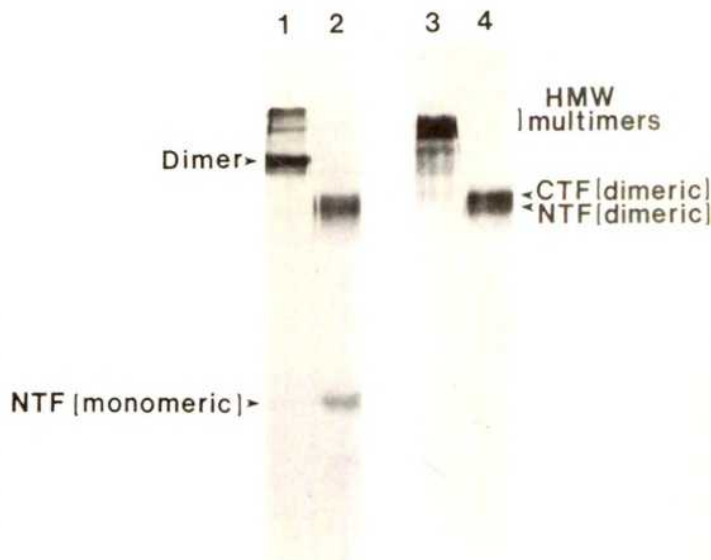


Fig 5. *Staphylococcus aureus* V-8 cleavage of multimeric vWf (autoradiograph of nonreduced 6% polyacrylamide gels). Lanes 1 and 2 show results obtained with constitutively secreted vWf obtained from long-term labeled human endothelial cell medium and lanes 3 and 4 show results with high-mol-wt (HMW) cellular vWf multimers. Lanes 1 and 3 represent the starting material, and lanes 2 and 4 show the cleavage products after V-8 protease digestion. Both CTF and NTF fragments obtained from the HMW multimers are dimeric, whereas the NTF obtained from culture medium vWf is mostly monomeric.

by amino acid sequence and carbohydrate content studies of the protein.²⁵ Both the N-terminal⁷ and C-terminal⁵⁻⁷ regions of the vWf polypeptide chain are rich in cysteines, but the center portion of the mature subunit contains a stretch of ~600 amino acids that is poor in cysteines,²⁶ similar to the disulfide distribution of fibronectin.²⁷ In addition, we showed that vWf dimers are similar to fibronectin dimers,^{28,29} in that both are held together at the C-terminal portion of the subunits (Fig 1, option B). In human cells, the formation of the vWf dimers is dependent on N-linked glycosylation of the subunits, since tunicamycin, which inhibits glycosylation, also blocks processing of vWf beyond the monomeric form.³

A recent abstract¹⁶ compared the electron microscopic appearance of N-terminal and C-terminal fragments of plasma vWf with that of the smallest circulating form of vWf and suggested that this dimeric molecule is held together in the C-terminal region of the subunits. It appears therefore that these small circulating species of vWf are not degradation products of large multimers but instead represent the dimeric molecules synthesized in the endoplasmic reticulum and secreted by endothelial cells^{2,23} and megakaryocytes³⁰ in culture.

Comparison of the nonreduced fragments obtained from dimeric and multimeric molecules (Figs 2 and 5) reveals that the interdimer disulfide bonds involve the N-terminal region of the subunit. This may occur by direct oxidation of the free sulfhydryl groups present on the pro vWf-dimers,² which are absent from mature vWf multimers.^{31,32} The multimerization process is likely to begin in the Trans Golgi apparatus³ and possibly continues during the packaging of the protein into the Weibel-Palade bodies. Loscalzo and colleagues showed that at high concentrations vWf can aggregate by noncovalent forces and proposed that such forces might be important during intracellular assembly of vWf.³³ We hypothesized that the large propolypeptide of vWf may assist in the proper alignment of the pro-vWf dimers for the interdimer disulfide bond formation.² The observation that these links are formed in proximity to the prosequence, ie, in the N-terminal region of the subunit, is further support for this hypothesis.

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Regulation of Early Human Hematopoietic (BFU-E and CFU-GEMM) Progenitor Cells In Vitro by Interleukin 1-Induced Fibroblast-Conditioned Medium

By James R. Zucali, Hal E. Broxmeyer, Charles A. Dinarello, Mary Ann Gross, and Roy S. Weiner

Stimulators of human erythroid burst-forming units (BFU-E) and multipotential colony-forming cells (CFU-GEMM) can be produced by a number of different cell types. A product of human peripheral blood monocytes, interleukin 1 (IL-1), was evaluated for its ability to stimulate fibroblast cultures to produce stimulators of human bone marrow BFU-E and CFU-GEMM colony formation. BFU-E and CFU-GEMM colony formation was evaluated using low-density, nonadherent low-density, and T lymphocyte-depleted nonadherent low-density human bone marrow cells cultured in the presence of a source of pure human erythropoietin. Both human monocyte conditioned medium (MCM) and human recombinant IL-1 (hrIL-1) induced fibroblasts to

produce stimulators of BFU-E and CFU-GEMM in a dose-dependent fashion with maximal colony formation occurring when fibroblasts were stimulated by 25% MCM or 140 ng/mL ROO6B hrIL-1, or 1.25 to 5 ng/mL ROO6T hrIL-1. Preincubation of MCM and hrIL-1 with an antibody to IL-1 inactivated the ability of MCM and hrIL-1 to induce the release of erythroid and multipotential colony stimulating activity from fibroblasts. These results suggest that monocyte-derived IL-1 is involved in regulating the production of humoral stimulators of early human hematopoietic progenitors.

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GROWTH OF HUMAN hematopoietic progenitor cells in clonal cultures requires the presence of specific soluble stimulating factors. Besides erythropoietin, erythroid burst-forming units (BFU-E) and multipotential (CFU-GEMM) progenitor cells require factors present in medium conditioned by blood leukocytes or other sources of burst promoting activity (BPA).^{1,2} A variety of cell types have been implicated in BPA production. These include monocytes and/or macrophages,^{3,4} T lymphocytes,^{5,6} and endothelial cells.⁷ Similar cell types have also been reported to be involved in GM-CSA production.⁸⁻¹⁰ Recent studies have focused on the monocyte as a central cell involved in the regulation of GM-CSA and BPA production.^{11,12} These studies report that monocytes produce a monokine or monokines which function by recruiting other accessory cells to produce GM-CSA and/or BPA.

We have recently identified interleukin 1 (IL-1) as one monocyte product which induces confluent fibroblast cultures to produce and release GM-CSA.¹³ Both purified human monocyte-derived IL-1 and human recombinant IL-1 (hrIL-1) reproduce the biologic activity found in this monocyte conditioned medium (MCM). In addition, a polyclonal antibody to IL-1 abolished this biologic activity. In the present study, we demonstrate that confluent fibroblast cultures, when stimulated by a factor present in MCM or by hrIL-1 also produce stimulators of colony formation by BFU-E and CFU-GEMM. Furthermore, the stimulation of fibroblast cultures by MCM or hrIL-1 can be blocked by the addition of antibody to IL-1.

MATERIALS AND METHODS

Isolation of peripheral blood monocytes. Peripheral blood cells were obtained from normal donors who had given informed consent. Peripheral blood leukocytes were isolated following semicontinuous flow pheresis¹⁴ and the buffy coat was diluted with two volumes of Hanks' balanced salt solution without calcium and magnesium prior to removing platelets by centrifugation at $150 \times g$ for ten minutes. This cell suspension was centrifuged over Ficoll-Hypaque (specific gravity 1.078) to remove contaminating granulocytes and erythrocytes. The mononuclear cells at the interface were collected and separated into two populations, monocytes and lymphocytes, by elutriation centrifugation as previously reported.^{13,15} Using this technique, monocytes were collected as a homogeneous cell population and further subjected to 2-aminoethylisothiuronium bromide

hydrobromide (AET)-treated sheep erythrocyte depletion to remove any contaminating T lymphocytes. Nonrosetting cells were separated by centrifugation through Ficoll-Hypaque. The purity of nonrosetting cells was determined using OKT3 (Ortho Diagnostics, Raritan, NJ) and LeuM3 (Becton Dickinson, Mountain View, CA) monoclonal antibodies and indirect immunofluorescence with affinity-purified goat antimouse IgG F(ab')₂-FITC (Cappel, West Chester, PA). The cells were analyzed using both fluorescence microscopy and a Coulter Model C flow cytometer. Background staining was determined on cells treated with nonimmune mouse immunoglobulins and second antibody. In addition, monocytes were identified on cytocentrifuge-prepared slides stained with Wright's-Giemsa and nonspecific esterase using alpha naphthyl acetate as substrate.¹⁶ The monocyte populations used in all experiments described contained <2% OKT3-positive, >98% LeuM3-positive, and >98% nonspecific esterase-positive cells.

Monocyte-conditioned medium preparation. Fresh or cryopreserved separated monocyte populations were cultured at 5×10^4 cells/mL for two days in alpha minimum essential medium (GIBCO, Grand Island, NY) containing 6% fetal bovine serum (Hyclone, Logan, UT) and 1 ng/mL endotoxin (LPS) (lipopolysaccharide B, *S. typhosa*, (DIFCO). The conditioned media were harvested and stored frozen at -20°C for assay.

Recombinant human IL-1. Human recombinant IL-1 (hrIL-1)

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containing amino acids 71 through 269 and having a molecular weight of 24.5 kD (ROO6B) or another recombinant preparation (ROO6T) containing amino acids 112 through 269 and having a molecular weight of 17.0 kD were obtained as previously described.^{13,17} Briefly, hrIL-1-producing bacteria were grown at 37 °C and lysed, and the hrIL-1 was purified by sequential ion exchange and either gel-filtration chromatography or high-performance liquid chromatography (HPLC). The hrIL-1 was stored at -70 °C in a 0.15 mol/L phosphate-buffered saline, pH 5.8. The identity of the purified hrIL-1 was confirmed by amino acid composition and sequence of the amino terminal heptapeptide. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as standard. Purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue or silver staining. The hrIL-1 was estimated to be greater than 99% pure.

Fibroblast conditioned medium preparation. Human lung fibroblasts (CCL 202) were obtained from the American Type Tissue Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (Hyclone). Fibroblasts from the first to the tenth passage were used, and the cell density ranged from 1 to 5 × 10⁴ cells per square centimeter. Cells were subcultured by brief (one-minute, 37 °C) treatment with 0.05% trypsin/0.02% disodium EDTA (wt/vol in PBS) and resuspended in fresh DMEM plus 10% fetal bovine serum and reseeded at a 1:5 dilution in 35-mm culture plates. At least 24 hours were allowed to elapse after initial seeding or subculture to obtain confluence before an experiment was begun. For assay, the fibroblast cell culture was replaced with 10% to 50% monocyte-conditioned medium, with or without a 1:100 dilution of the IgG fraction of rabbit antihuman IL-1,¹⁸ various concentrations of hrIL-1, or control medium diluted with fibroblast culture medium (FCM), and the cultures were returned to the incubator for an additional 24 hours. As controls, hrIL-1 alone, MCM alone, and FCM in the absence of hrIL-1 or MCM were incubated for 24 hours prior to assay. After 24 hours, the medium was aspirated and centrifuged (200 × g, five minutes), and the supernatants stored at -20 °C until tested.

Bone marrow cell preparation. Bone marrow cells were obtained by aspiration from the posterior iliac crest of healthy volunteers who had given informed consent. Unseparated nucleated buffy coat cells were obtained by centrifugation of whole bone marrow aspirates at 1,500 rpm for ten minutes, and isolation of the white cells that overlay the red cell layer. Low-density (LD) cells (1.077 g/mL) were obtained after separation on Ficoll-Hypaque (Pharmacia, Uppsala) at 1,500 rpm for 30 minutes, washed three times, and resuspended in Iscove's modified Dulbecco medium (IMDM) (GIBCO) containing 10% fetal bovine serum. Cells were further separated into nonadherent low-density (NALD) cells after incubation on plastic tissue culture dishes (Falcon, Oxnard, CA) for 90 minutes at 37 °C and 5% CO₂. NALD cells were collected by gently swirling the dishes and slowly removing the suspension cells. NALD cells prepared in this manner routinely contained <2% nonspecific esterase-positive cells. To deplete T lymphocytes, NALD cells were further separated into E-rosette-positive (E+) populations according to a method modified from Weiner et al¹⁹ as described previously.²⁰ Nonrosetting cells were separated by centrifugation through Ficoll-Hypaque. The purity of the NALD T lymphocyte-depleted fraction (NALDT⁻) was analyzed by indirect immunofluorescence with OKT3 or OKT11a as described above. The NALDT⁻ marrow cell fraction contained <2% OKT11a-positive cells.

BFU-E and CFU-GEMM assay. Bone marrow cells were plated at 1 × 10⁵ for LD and NALD cells and 4 × 10⁴ for NALDT⁻ cells in 35-mm Lux standard tissue culture dishes containing a 1-mL mixture of IMDM, 1% methylcellulose, 30% FBS, and 5 × 10⁻⁵ mol/L 2-mercaptoethanol. Cultures contained 0.1 mmol/L bovine hemin (Eastman Kodak Co, Rochester, NY) for CFU-GEMM colonies, while BFU-E were cultured without hemin.^{20,21} Human urinary erythropoietin (Toyoba, New York) or recombinant erythropoietin (Amgen, Thousand Oaks, CA) 1 unit/mL and the conditioned mediums to be tested were added to the dishes on day 0. Results were comparable using the two preparations of erythropoietin. Dishes were incubated at 37 °C in a humidified atmosphere flushed with 5% CO₂ and lowered (5%) O₂ for 14 days, at which time colonies were scored for BFU-E and CFU-GEMM. Lowered oxygen

Table 1. Influence of FCM (MCM) and FCM (IL-1) on Colony Formation From BFU-E and CFU-GEMM by 10⁵ Low-Density Normal Human Bone Marrow Cells (LD)

Additions	BFU-E* Experiments		CFU-GEMM† Experiments	
	1	2	1	2
Control medium	56 ± 2‡	32 ± 2	0	0
MCM§ (25%)	69 ± 2		3 ± 1	
MCM (50%)		32 ± 4		0
hrIL-1 (350 ng/mL)	58 ± 3	27 ± 2	0	0
FCM (control)	53 ± 2	30 ± 3	0	0
FCM (MCM 10%)	77 ± 3		7 ± 1	
FCM (MCM 25%)	98 ± 1		11 ± 1	
FCM (MCM 50%)		51 ± 3		6 ± 1
FCM (hrIL-1 350 ng/mL)¶	95 ± 7	59 ± 2	9 ± 1	7 ± 1
FCM (hrIL-1 140 ng/mL)	77 ± 2		8 ± 1	
FCM (hrIL-1 70 ng/mL)	73 ± 5		5 ± 1	
FCM (hrIL-1 35 ng/mL)	56 ± 4		1 ± 1	
FCM (control) + MCM (50%)	29 ± 1		0	
FCM (MCM 50%) + FCM (Control)		53 ± 4		7 ± 1
FCM (MCM 50%) ± MCM (50%)	55 ± 1		6 ± 0	

*BFU-E were cultured in the presence of 1 unit/mL erythropoietin (Epo) without hemin.

†CFU-GEMM were cultured in the presence of 1 unit/mL erythropoietin (Epo) and 0.1 mmol/L hemin.

‡Results were expressed as mean ± 1 SEM of four or five replicate cultures.

§Monocyte-conditioned medium.

||Resulting fibroblast-conditioned medium after stimulation by monocyte-conditioned medium.

¶Resulting fibroblast-conditioned medium after stimulation by human recombinant IL-1.

Table 2. Influence of FCM (MCM) and FCM (hrIL-1) on Colony Formation From BFU-E and CFU-GEMM by 10^6 Nonadherent Low-Density Normal Human Bone Marrow Cells (NALD)

Additions	BFU-E* Experiments		CFU-GEMM† Experiments	
	1	2	1	2
Control medium	48 ± 3‡	39 ± 2	0	0
MCM§ 50%	62 ± 3	45 ± 5	0	0
hrIL-1 350 ng/mL	50 ± 2	40 ± 1	0	0
FCM (Control)	49 ± 1	40 ± 2	0	0
FCM (MCM 10%)	91 ± 8	89 ± 9	14 ± 1	5 ± 0
FCM (MCM 25%)	134 ± 2	100 ± 1	22 ± 2	13 ± 3
FCM (MCM 50%)	110 ± 9	82 ± 3	27 ± 4	15 ± 2
FCM (MCM 25% + ab IL-1)¶	63 ± 1	38 ± 1	0	0
FCM (hrIL-1# 350 ng/mL)	101 ± 5	67 ± 2	36 ± 1	15 ± 2
FCM (hrIL-1 140 ng/mL)	101 ± 5	92 ± 5	27 ± 2	21 ± 2
FCM (hrIL-1 70 ng/mL)	87 ± 5	77 ± 3	15 ± 1	1 ± 0
FCM (hrIL-1 35 ng/mL)	77 ± 5	55 ± 2	6 ± 2	0
FCM (hrIL-1 70 ng/mL + ab IL-1)**	54 ± 4	42 ± 1	0	1 ± 0

* , † , ‡ , § , || See footnotes, Table 1.

¶ Resulting fibroblast conditioned medium after stimulation by monocyte-conditioned medium previously incubated with antibody to IL-1.

See Table 1, footnote ¶.

** Resulting fibroblast-conditioned medium after stimulation by hrIL-1 previously incubated with antibody to IL-1.

tension was obtained using an oxyreducer (Reming Bioinstruments, NY).²¹ The criteria for evaluating BFU-E colonies have been given elsewhere.^{20,21} Mixed (CFU-GEMM) colonies contained erythroid cells with granulocytes and macrophages and sometimes megakaryocytes.

Statistical analysis. Levels of significance for comparisons between samples were determined using the Student's *t* distribution. The results are expressed as mean ± 1 standard error of the mean (SEM) of four or five plates per point for each experiment.

RESULTS

Both human monocyte-conditioned medium (MCM) and human recombinant interleukin 1 (hrIL-1) were shown to induce confluent fibroblast cultures to produce and release

stimulators of BFU-E and CFU-GEMM in nine different experiments using seven different target bone marrow preparations. Stimulation occurred whether the target bone marrow was depleted of high-density cells (Table 1, two separate experiments using two different target bone marrow populations), high-density cells plus adherent cells (Table 2, two separate experiments using two different target bone marrow cells), or high-density cells plus adherent cells plus T lymphocytes (Table 3, five different experiments using three different target bone marrow cells). Colony growth was significantly greater ($P < 0.005$) when bone marrow cultures were stimulated by supernatants of fibroblasts cultured in the presence of MCM or hrIL-1 when compared to supernatants of fibroblasts or monocytes cultured in medium alone.

Table 3. Influence of FCM (MCM) and FCM (hrIL-1) on Colony Formation From BFU-E and CFU-GEMM by 4×10^4 Nonadherent Low-Density T Cell-Depleted Human Bone Marrow Cells (NALDT)

Additions	BFU-E* Experiments			CFU-GEMM† Experiments		
	1	2	3	1	2	3
Control medium	27 ± 1‡	46 ± 1	45 ± 4	0	2 ± 1	1 ± 0
MCM§ 50%	26 ± 1	49 ± 2	46 ± 2	0	2 ± 1	2 ± 0
hrIL-1 1X	27 ± 1	48 ± 2	47 ± 2	0	2 ± 0	1 ± 1
FCM (Control)	29 ± 1	51 ± 2	47 ± 2	0	2 ± 1	2 ± 0
FCM (MCM 10%)	103 ± 1			17 ± 2		
FCM (MCM 25%)	124 ± 5	96 ± 7	99 ± 5	31 ± 3	24 ± 2	27 ± 1
FCM (MCM 50%)	90 ± 6			33 ± 2		
FCM (MCM 25% + ab IL-1)¶	37 ± 2	48 ± 1	47 ± 2	0	3 ± 1	2 ± 1
FCM (hrIL-1# 1X)	93 ± 12	86 ± 4	113 ± 6	25 ± 5	32 ± 2	29 ± 2
FCM (hrIL-1) 1:2	118 ± 8	95 ± 2	103 ± 6	31 ± 3	29 ± 1	28 ± 2
FCM (hrIL-1) 1:4	98 ± 6	92 ± 7	63 ± 3	18 ± 5	19 ± 2	14 ± 1
FCM (hrIL-1) 1:8	47 ± 4	67 ± 2	47 ± 2	3 ± 1	12 ± 1	9 ± 1
FCM (hrIL-1) 1:2 (+ IL-1)**	33 ± 1	46 ± 2	45 ± 2	0	2 ± 0	2 ± 1

* , † , ‡ , § , || See footnotes, Table 1.

¶ Resulting fibroblast-conditioned medium after stimulation by monocyte-conditioned medium previously incubated with antibody to IL-1.

Experiment 1, human recombinant IL-1 (a 24.5-kD molecule containing amino acids 71 through 269) 1x = 350 ng/mL. Experiments 2 and 3, human recombinant IL-1 (a 17.0-kD molecule containing amino acids 112 through 269) 1x = 5 ng/mL.

** Resulting fibroblast-conditioned medium after stimulation by human recombinant IL-1 previously incubated with antibody to IL-1.

Erythroid BFU-E colonies stimulated with FCM(MCM) or with FCM(hrIL-1) were much larger in size than the erythroid colonies in the absence of FCM(MCM) or FCM(hrIL-1). The effects of MCM or hrIL-1 required intact fibroblasts, since mixtures of FCM and MCM contained no more stimulatory activity than FCM or MCM alone (Group 13, Table 1). In the absence of Epo or Epo plus hemin, FCM(MCM) or FCM(hrIL-1) did not stimulate any BFU-E or CFU-GEMM colonies, but they did stimulate numerous CFU-GM colonies. Thus, in experiment 1 reported in Table 2, FCM(MCM 25%) stimulated 112 ± 3 day 7 and 64 ± 1 day 14 CFU-GM colonies while FCM(hrIL-1, 140 ng/mL) stimulated 108 ± 3 day 7 and 59 ± 4 day 14 CFU-GM colonies, respectively. Similar results were previously reported for the stimulation of CFU-GM by IL-1-induced fibroblast-conditioned medium.¹³

To rule out the possibility that monocyte-conditioned medium or fibroblast-conditioned medium contained inhibitors for BFU-E and CFU-GEMM growth which were inactivated, allowing for the expression of enhanced colony growth, the following study was performed (Table 4). We assessed the effects of MCM, FCM and FCM(MCM) on erythroid and mixed colony formation stimulated by Epo or Epo plus hemin in the presence of 5637-conditioned medium. Medium conditioned with 5637 has been shown to be appropriate for the detection of suppressor factors.²² As seen in Table 4, none of the conditioned medium-suppressed colony formation by 5637-conditioned medium and the FCM(MCM) actually enhanced colony formation to a level equal to or slightly higher than that of FCM(MCM) in the absence of 5637-conditioned medium. (Compare group 6, experiments 2 and 3, Table 3 with group 4, Table 4).

Maximal colony formation by BFU-E and CFU-GEMM occurred when fibroblast cultures were stimulated by 70 to 140 ng/mL hrIL-1 (24.5 kD), 1.25 to 5.0 ng/mL hrIL-1 (17.0 kD) or 25% to 50% MCM. Conditioned medium obtained from fibroblasts cultured in the presence of increasing concentrations of hrIL-1 (35 to 140 ng/mL or 0.625 to 5.0 ng/mL) and MCM (10% to 25%) resulted in dose-

response increases in colony formation by BFU-E and CFU-GEMM.

As further proof that the activity found in monocyte-conditioned medium was due to IL-1 production by monocytes, studies were conducted using an antibody to IL-1. A 1:100 dilution of anti-IL-1 completely abolished the stimulation of CFU-GEMM by FCM(MCM 25%), while significantly reducing the enhancement of BFU-E colony formation. This same antibody completely abolished the stimulation of CFU-GEMM and BFU-E seen with fibroblast-conditioned medium collected in the presence of hrIL-1 (Tables 2 and 3). In addition, using this same IL-1 antibody, we tested to see if background erythroid colony formation stimulated in the presence of Epo or Epo plus hemin could be reduced by preincubation of the cells with IL-1 antibody. Cells were incubated for one hour with antibody to IL-1 (1:100 final dilution) in the presence of Epo or Epo plus hemin, or were incubated for one hour with McCoy's medium in the presence of Epo or Epo plus hemin. The cells were then plated without washing so that the antibody to IL-1 or control medium were plated also. Erythroid growth and CFU-GEMM growth were unchanged (BFU-E 63 ± 3 v 64 ± 3 and CFU-GEMM 5 ± 1 v 5 ± 1) when cells were incubated in the absence or presence of antibody to IL-1, respectively.

DISCUSSION

Assays are available to detect early human clonogenic progenitors that give rise to large erythroid colonies (BFU-E),¹ and colonies containing erythroid cells, granulocytes, megakaryocytes, and macrophages (CFU-GEMM).² Growth of these early progenitor cells depends on the presence of a source of burst-promoting activity (BPA) or conditioned medium obtained from leukocytes of patients with hemochromatosis cultured in the presence of 1% phytohemagglutinin. A number of different cell types including monocytes or macrophages,^{3,4} T lymphocytes,^{5,6} and endothelial cells⁷ have been reported to produce BPA. Recent studies on regulatory cell interactions have reported that monocytes produce a factor (monokine) which stimulates production of BPA by endothelial cells.¹² A similar cell-to-cell relationship has been proposed for GM-CSA production¹¹ and we have recently identified interleukin 1 (IL-1) as one monocyte product which stimulates fibroblasts to produce GM-CSA.¹³ The results of the present study show that fibroblasts are also capable of producing stimulating activities for BFU-E and CFU-GEMM colony formation. The production of this stimulating activity by fibroblasts requires prior induction by a monocyte product which we have identified as IL-1. Fibroblasts cultured in the presence of monocyte-conditioned medium or human recombinant IL-1 stimulated significantly greater BFU-E and CFU-GEMM colony growth when compared to supernatants of fibroblasts, monocytes, or IL-1 cultured alone. Both FCM(MCM) and FCM(hrIL-1) stimulated BFU-E and CFU-GEMM colony formation in a dose-dependent fashion with maximal colony formation occurring when fibroblasts were stimulated by 25% to 50% MCM, 70 to 140 ng/mL hrIL-1 (ROO6B), or 1.25 to 5

Table 4. Assessing MCM, FCM, and the Resulting Fibroblast-Conditioned Medium After Stimulation by Monocyte-Conditioned Medium [FCM(MCM)] for Inhibitory Activity Against Colony Formation by BFU-E and CFU-GEMM.*

Additions to 5637 CM†	BFU-E‡ Experiments		CFU-GEMM§ Experiments	
	1	2	1	2
Control medium	107 ± 4	108 ± 7	8 ± 1	8 ± 0
MCM	105 ± 4	109 ± 4	9 ± 1	9 ± 1
FCM	106 ± 3	113 ± 8	9 ± 1	9 ± 1
FCM (MCM)	112 ± 4	116 ± 6	28 ± 2	27 ± 2

*Similar results were seen with a second target bone marrow in two separate experiments.

†5637 human bladder carcinoma-conditioned medium.

‡BFU-E were cultured in the presence of 1 unit/mL Toyoba Epo and 0.1 mmol/L hemin.

§CFU-GEMM were cultured in the presence of 1 unit/mL Toyoba Epo and 0.1 mmol/L hemin.

||Results are expressed as mean $\pm$ 1 SEM of four plates per point.

ng/mL hrIL-1 (ROO6T). In addition, a polyclonal rabbit antibody specific for human monocyte IL-1 completely abolished CFU-GEMM colony-stimulating activity while completely reducing the enhancing activity for BFU-E by FCM(hrIL-1) and almost completely reducing the enhancing activity for BFU-E by FCM(MCM).

The presence of inhibitors in monocyte-conditioned medium or fibroblast conditioned medium to erythroid and mixed colony growth appear unlikely, since neither MCM nor FCM suppressed colony growth stimulated by 5637 human bladder carcinoma-conditioned medium. The action of FCM(MCM) and FCM(hrIL-1) on BFU-E and CFU-GEMM colony growth do not appear to require monocytes or T lymphocytes in the marrow population containing BFU-E and CFU-GEMM, since results were similar when the target bone marrow population was depleted of both adherent cells and T lymphocytes.

Because FCM(hrIL-1) contains stimulators for CFU-GM, BFU-E, and CFU-GEMM, it is possible that all of

these actions are brought about by the presence of one or more factors. In this regard, human recombinant GM-CSF has been shown to stimulate BFU-E as well as CFU-GM.²³ Similarly, a pluripotent hematopoietic colony-stimulating factor (pluripotent CSA) has been purified from conditioned medium of the human bladder carcinoma cell line 5637.²⁴ This pluripotent CSF supports the growth of human mixed colonies, granulocyte-macrophage colonies, and early erythroid colonies and induces differentiation of the human promyelocytic leukemic cell line HL-60 and the murine myelomonocytic leukemic cell line WEHI-3B (D+). Whether the resulting fibroblast-conditioned medium stimulated by IL-1 and reported here contains one or more of these CSFs must await purification studies.

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Platelet Aggregation in Whole Blood From Patients With Glanzmann's Thrombasthenia

By Michael E. Burgess-Wilson, Stephen R. Cockbill, Geoffrey I. Johnston, and Stanley Heptinstall

We examined platelet aggregation in platelet-rich plasma (PRP) and in whole blood from two patients with Glanzmann's thrombasthenia. In PRP, aggregation was measured by monitoring the changes in light absorbance that occurred in response to aggregating agents; to measure platelet aggregation in whole blood, we used a platelet counting technique. In PRP, the patients' platelets showed defective aggregation in response to ADP, adrenaline, arachidonic acid (AA), and collagen, but normal agglutination occurred in response to ristocetin. In whole blood, however, platelet aggregation in response to the aggregating agents appeared to be either very similar to that which

occurred in blood from normal subjects or only slightly reduced. There was a reduced response to all concentrations of ADP and to low concentrations of collagen but a normal response to all concentrations of adrenaline, AA, and higher concentrations of collagen. Conversely, there seemed to be an increased agglutination response to ristocetin. The abnormality in our two patients with Glanzmann's thrombasthenia probably lies in the inability of their platelets to form large, macroscopic aggregates rather than in platelet aggregation *per se*.

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GLANZMANN'S thrombasthenia is a rare bleeding disorder characterized by defective platelet aggregation but with a normal platelet count.¹ Platelets from patients with this condition either do not aggregate or aggregate very poorly in response to agents such as ADP, adrenaline, arachidonic acid (AA), and collagen. Platelets from such patients agglutinate normally in response to ristocetin,^{2,3} and this has been used to aid the diagnosis of the condition. Defective platelet aggregation is believed to result from a markedly reduced amount of a complex of glycoproteins (the glycoprotein IIb/IIIa complex) on the platelet surface.⁴ This glycoprotein complex is essential for normal platelet aggregation but plays no part in ristocetin-induced platelet agglutination.

Previous studies in which platelet aggregation in Glanzmann's thrombasthenia was compared with platelet aggregation in healthy controls involved the use of platelet-rich plasma (PRP), in which aggregation is measured by following the changes in light absorbance that occur. With this technique, macroscopic platelet aggregates must form before the light absorbance of the PRP falls. It is now possible to measure platelet aggregation in whole blood by counting the number of single platelets (ie, unaggregated platelets) using a whole-blood platelet counter.^{5,6} Because the technique relies on the determination of the number of single platelets that remain in the blood rather than the size of the platelet aggregates that form, it is an extremely sensitive means of detecting platelet aggregates composed of relatively few platelets. In the present investigation, we compared platelet aggregation in PRP (measured with light absorbance) and

whole blood (measured with the platelet counting technique) from patients with Glanzmann's thrombasthenia. The results obtained using whole blood were compared with those obtained for healthy controls.

MATERIALS AND METHODS

Materials. Adenosine 5'-diphosphate (ADP, sodium salt) adrenaline, and arachidonic acid (AA, grade 1 from porcine liver) were obtained from Sigma Chemical (St Louis). ADP and adrenaline were dissolved in saline before use; AA was converted to the sodium salt before use by dissolving it in 0.1 mol/L of sodium carbonate. Collagen (suspended equine collagen fibrils) was obtained from Hormon-Chemie and used as directed. Ristocetin sulphate was obtained from H. Lundbeck and dissolved in saline. Saline was 150 mmol/L of sodium chloride from Travenol Laboratories. The solution of citrate that was used to anticoagulate blood was 3.8% (wt/vol) trisodium citrate dihydrate. M148 (a monoclonal antibody directed against the glycoprotein IIb/IIIa complex) was a gift from Professor R.M. Hardisty, Institute of Child Health, London, and was used at a final dilution of 1:2,000. A control murine antibody 791T/36 (human osteogenic sarcoma cell-line) was a gift from Dr M. Price, Cancer Research Laboratories, University of Nottingham, England.

Subjects. Blood samples were taken from a sister and brother, designated as patient 1 and patient 2 throughout, aged 17 and 19 years, respectively, who had a similar history of mild bruising and bleeding. The diagnosis of Glanzmann's thrombasthenia had been made by Dr F.E. Preston (Department of Haematology, Royal Hallamshire Hospital, Sheffield, England) on the basis of clinical history and the pattern of platelet aggregation in PRP characteristic of the condition. When examined, the patients exhibited prolonged bleeding times (30 and 21 minutes, respectively), but clot retraction was normal as were the whole blood platelet counts.

Blood samples were also taken from six healthy controls (three men and three women with an age range of 18 to 42 years) to obtain a set of normal values for platelet aggregation in whole blood. The tests on these samples were carried out using the same protocol as that used for the two patients.

Blood collection. Blood was collected with minimum venous stasis from the antecubital vein into polypropylene syringes using a 19 gauge, 2-inch needle, and aliquots (9 mL) were dispensed into polystyrene tubes that contained 1 mL of citrate. Some of the citrated blood was centrifuged at 180 g for 10 minutes to prepare PRP, and the blood from which the PRP had been removed was then centrifuged at 1,200 g for 20 minutes to obtain platelet-poor plasma. The latter was used to adjust the platelet count in the PRP to 300 × 10⁹ platelets/L.

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Measurements of platelet aggregation. Platelet aggregation in PRP was studied using the aggregometer and technique described by Adams and colleagues.⁷ Changes in light absorbance were recorded using a pen recorder.

Platelet aggregation in whole blood was assessed by determining the number of single platelets that remained in the blood after adding an aggregating agent to a sample. The number of single platelets present was counted using an Ultra-Flo 100 Whole Blood Platelet Counter and the technique described by Fox and co-workers.⁶ In brief, samples (500 μ L) of blood were placed in small polystyrene tubes, and the tubes were placed in a water bath at 37 °C. After 2 minutes, a small aliquot of the blood was removed for platelet counting, a metal stirrer bar was placed in each tube, and the blood was stirred at 1,000 rpm. After a further 2 minutes, another small aliquot was removed for platelet counting, and a solution of the aggregating agent under investigation was then added. Stirring was then continued, and further small aliquots were removed for platelet counting at 2-minute intervals up to 6 minutes after addition of the aggregating agent. The results obtained were expressed as the percentage of single platelets lost from the blood relative to the number of platelets present immediately prior to the addition of aggregating agent.

Inhibition of platelet aggregation by M148. The monoclonal antibody, M148, binds to the glycoprotein IIb/IIIa complex on the surface of normal platelets and inhibits aggregation induced by ADP, adrenaline, AA, and collagen in both PRP and in whole blood.⁸ In some experiments, M148 (at a final dilution of 1:2,000) was added to blood from the two patients with Glanzmann's thrombasthenia 2 minutes before the aggregating agent; aliquots were then removed for platelet counting at 20-second intervals up to 2 minutes after addition of the aggregating agent. To facilitate this quicker subsampling technique, the aliquots (15 μ L) were taken into fixative as described by Bevan and colleagues⁹ to be counted later on the Ultra-Flo 100. The results were compared with those obtained using a control murine antibody (791T/36).

Measurement of glycoprotein expression. Expression of the glycoprotein IIb/IIIa complex on the surface of platelets from the patients was measured using the monoclonal antibody, M148,¹⁰ combined with the technique of flow cytofluorimetry. After 240 μ L samples of PRP were incubated with M148, aliquots containing 2×10^6 platelets were removed to tubes containing a fluorescent-labeled rabbit anti-mouse immunoglobulin as described previously.¹¹ After a further incubation, immunofluorescence analysis was carried out using the FACS IV Flow Cytofluorimeter.¹²

Microscopic examination of platelet aggregates. After samples of blood from both patients with Glanzmann's thrombasthenia and samples of blood from a healthy control were stirred with ADP (10 μ mol/L), adrenaline (10 μ mol/L), AA (0.2 mmol/L), collagen (6 μ g/mL), and ristocetin (2 mg/mL), each for 6 minutes, the blood was smeared on a glass slide and stained with May-Grunwald-Giemsa stain. The tails of the blood smears were then examined for platelet/platelet aggregates under a light microscope. The effect of preincubating blood from the patients and controls with M148 or control antibody prior to adding 5 μ mol/L of ADP was also determined.

RESULTS

Platelet aggregation in PRP. The light absorbance of samples of PRP from the patients with Glanzmann's thrombasthenia either did not fall or fell only slightly when adrenaline, ADP, AA, or collagen was added to the PRP. In contrast, adding ristocetin to PRP from the patients induced a large fall in light absorbance; this was accompanied by the appearance of macroscopic platelet aggregates in the sample.

Examples of the results obtained for the two patients are given in Fig 1, these results having been seen on more than one occasion.

Platelet aggregation in whole blood. In contrast to the results obtained in PRP, platelet aggregation did occur when ADP, adrenaline, AA, or collagen was added to whole blood from the patients with Glanzmann's thrombasthenia. Table 1 shows the percentage loss of single platelets 6 minutes after the addition of aggregating agents to samples of whole blood obtained from the two patients and the controls. There was a reduced response in both patients, as compared with the mean control value, to all concentrations of ADP (Fig 2) and to the lower concentrations of collagen (0.3 and 1.0 μ g/mL) and AA (0.1 mmol/L) but a normal response to all concentrations of adrenaline and higher concentrations of collagen (3 and 6 μ g/mL) and AA (0.2 mmol/L). There was an increased agglutination response to the lower concentration of ristocetin used.

When samples of whole blood from the patients with Glanzmann's thrombasthenia were preincubated with M148, the platelet aggregation induced by ADP, adrenaline, and collagen, was virtually abolished (Fig 3).

Glycoprotein expression. After immunofluorescence analysis of platelets incubated with M148 followed by a fluorescent-labeled immunoglobulin, the number of glyco-

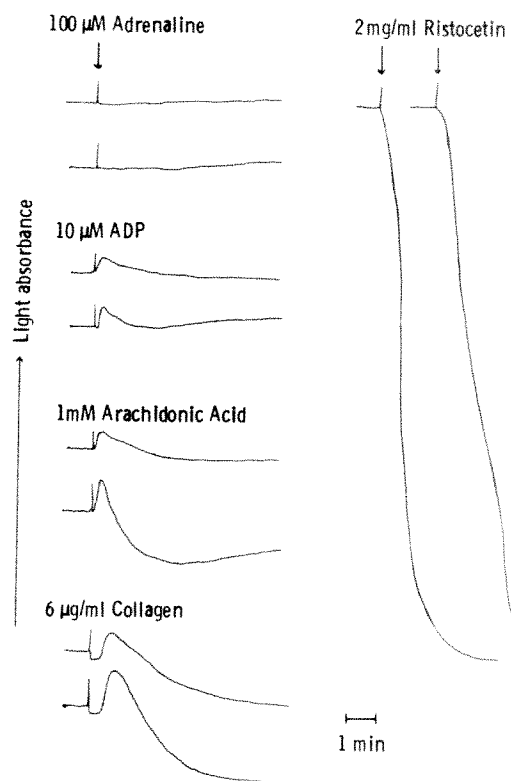


Fig 1. The changes in light absorbance that occurred in platelet-rich plasma (PRP) (480 μ L) that had been stirred at 37 °C for 2 minutes followed by the addition of various aggregating agents (20 μ L). Typical traces obtained from both of the patients on one occasion are shown. For each aggregating agent, the first of each pair of traces is that obtained for patient 2 and the second is that for patient 1.

Table 1. Percentage of Single Platelets Lost Six Minutes After Addition of Aggregating Agents to Samples of Whole Blood Stirred at 37 °C

Aggregating Agent	Patient 1	Patient 2	Controls
Saline	2	4	10 ± 6
ADP (0.5 µmol/L)	9	8	22 ± 5
ADP (1.0 µmol/L)	4	6	30 ± 14
ADP (2.0 µmol/L)	26	15	52 ± 22
ADP (10.0 µmol/L)	40	28	90 ± 9
Adrenaline (0.2 µmol/L)	16	10	10 ± 7
Adrenaline (0.5 µmol/L)	18	19	16 ± 9
Adrenaline (1.0 µmol/L)	21	25	20 ± 9
Adrenaline (2.0 µmol/L)	24	38	26 ± 10
Adrenaline (5.0 µmol/L)	30	39	33 ± 17
AA (0.1 mmol/L)	4	2	43 ± 33
AA (0.2 mmol/L)	92	93	95 ± 3
Collagen (0.3 µg/mL)	11	7	20 ± 14
Collagen (1.0 µg/mL)	31	18	80 ± 29
Collagen (3.0 µg/mL)	89	90	96 ± 1
Collagen (6.0 µg/mL)	92	94	97 ± 1
Ristocetin (1.0 mg/mL)	96	99	20 ± 13
Ristocetin (2.0 mg/mL)	97	99	95 ± 2

Control values are mean ± SD (n = 6).

protein IIb/IIIa complexes on the surface of platelets from patients 1 and 2 were calculated to be 450 and 1,600, respectively. This compares with a mean value of 17,300 for platelets from normal subjects.⁸ We have already shown that these patients have platelets that are deficient in glycoprotein

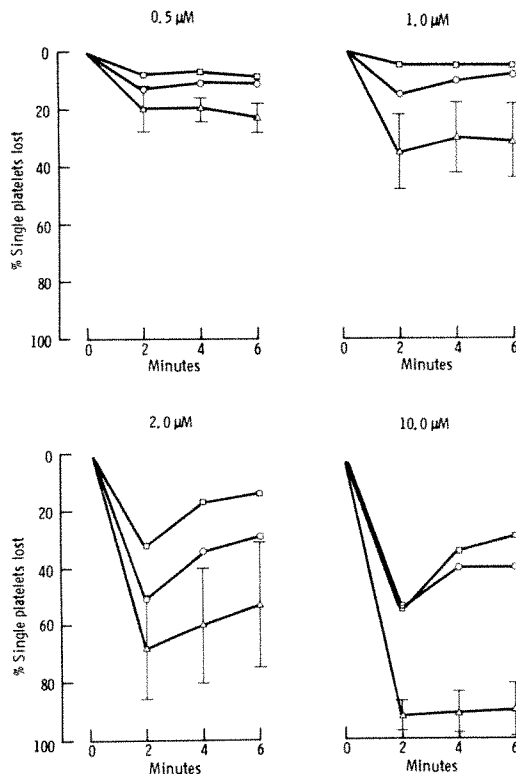


Fig 2. The percentage of single platelets lost from whole blood stirred at 37 °C following the addition of different concentrations of ADP at time 0. The results shown are for patient 1 (○) and patient 2 (□). The control values (Δ) are mean ± SD (n = 6).

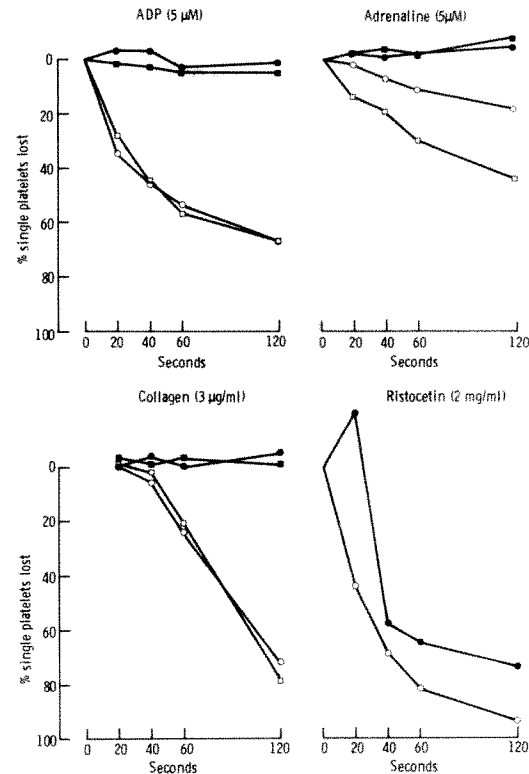


Fig 3. The percentage of single platelets lost from whole blood stirred at 37 °C following the addition of various aggregating agents at time 0. The blood had been preincubated for 2 minutes with either M148 or a control antibody. The results are for patient 1 in the presence of either M148 (●) or control antibody (○) and for patient 2 in the presence of either M148 (■) or control antibody (□). Unfortunately, only data for patient 1 were obtained for ristocetin-induced agglutination.

IIb/IIIa and that the deficiency does not reside in a subpopulation of cells.¹¹

Microscopy. Samples of blood to which aggregating agents had been added were examined for the presence of platelet aggregates by light microscopy. Platelet aggregates were present in whole blood from the patients with Glanzmann's thrombasthenia as well as in blood from the normal control. When blood from the patients or controls was incubated with M148 before adding ADP, only single platelets were seen.

DISCUSSION

The results of our studies on platelet aggregation in PRP in which we used light absorption as a means of measuring the platelet aggregation that occurred are entirely consistent with those obtained by other workers who have investigated patients with Glanzmann's thrombasthenia.¹⁻³ Aggregation was clearly defective in PRP from the two patients that we investigated. We were therefore surprised to find that the loss of single platelets induced by the addition of aggregating agents to whole blood from the patients appeared to be very similar to the loss obtained from healthy controls or was only slightly reduced. The reduced response to ADP was >1 SD at lower concentrations and >2 SDs at the highest concentra-

tion ($10 \mu\text{mol/L}$), whereas the reduced responses to collagen and AA were only seen at the lower concentrations tested.

By preincubating whole blood from the patients with M148, we were able to confirm that the single platelets that were lost when an aggregating agent was added to whole blood were indeed taking part in aggregate formation. The addition of this monoclonal antibody virtually abolished the platelet loss that occurred following addition of ADP, adrenaline, or collagen to blood from the patients with Glanzmann's thrombasthenia. As further confirmation that platelet aggregation was occurring in whole blood, we looked directly for platelet aggregates in blood from the patients using light microscopy. Platelet aggregates were observed in all the samples to which an aggregating agent had been added. In addition, M148 prevented aggregate formation.

We consider that the apparent discrepancy between results obtained in whole blood and PRP in this investigation relates to the differences in the two techniques that were used to measure the aggregation that occurred. The light absorbance of PRP falls only when very large platelet aggregates (that are visible to the naked eye and must contain many thousands of platelets) form. Conversely, the number of single platelets in whole blood falls as soon as the single platelets take part in aggregate formation whatever the size of the aggregates that develop. The defective platelet aggregation in PRP from patients with Glanzmann's thrombasthenia may occur because the aggregates that form are relatively small. We believe that it is unlikely that the different results in PRP and whole blood relate to the presence of RBCs in the latter, but this possibility has not been excluded.

Although the patients we investigated had platelets that contained markedly reduced amounts of surface-expressed glycoprotein IIb/IIIa complex (3% and 9% of normal for patients 1 and 2, respectively), the platelets were not completely devoid of the glycoprotein complex. In addition, clot retraction occurred in coagulated whole blood from these patients. Thus, according to the definition of Caen,¹³ our patients probably have type II Glanzmann's thrombasthenia. Platelets from patients with type I Glanzmann's thrombasthenia have undetectable amounts of the glycoprotein complex and clot retraction is absent in coagulated blood.

Although Nurden and colleagues¹⁴ recently reported the presence of measurable amounts of glycoprotein IIb and/or glycoprotein IIIa on the platelets of patients with type I Glanzmann's thrombasthenia, they proposed that it was the number of functional glycoprotein IIb/IIIa complexes expressed on the surface of the platelet that was important in discriminating between the two subgroups. It is probable that the small amounts of complex that are present on our patients' platelets allow partial platelet aggregation that is detectable in our whole blood system but not in PRP. In view of the reduced platelet aggregation that occurs in response to ADP and low concentrations of collagen in whole blood from these patients, it will be interesting to discover if platelet aggregation is further reduced in whole blood from patients with a more severe glycoprotein IIb/IIIa complex deficiency. It must be noted that in our investigation platelets from patient 1 aggregated slightly more extensively in both PRP and whole blood than did platelets from patient 2, despite having a lesser amount of the glycoprotein IIb/IIIa complex available and a longer bleeding time. This lack of correlation between glycoprotein expression, platelet aggregation, and bleeding time in these two patients may be caused by the close similarity of the patients with regard to both clinical and laboratory parameters, however.

In the cases of Glanzmann's thrombasthenia we have studied, only relatively small amounts of glycoprotein IIb/IIIa were expressed, and the deficiency resides in all the platelets rather than in a subpopulation.¹¹ We have suggested that it is the amount of glycoprotein IIb/IIIa on the surface of platelets that determines the size of the aggregates that are produced in response to an aggregating agent and that the true deficiency in Glanzmann's thrombasthenia relates to the size of the aggregates produced rather than to aggregation per se. The precise relationship between aggregate size and glycoprotein IIb/IIIa expression needs to be explored by further experimentation.

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Purification and Properties of Bacterially Synthesized Human Granulocyte-Macrophage Colony Stimulating Factor

By Antony W. Burgess, C. Glenn Begley, Gregory R. Johnson, Angel F. Lopez, D. James Williamson, Jean Jacques Mermod, Richard J. Simpson, Albert Schmitz, and John F. DeLamarter

Human granulocyte-macrophage colony stimulating factor (GM-CSF) has been synthesized in high yield using a temperature inducible plasmid in *Escherichia coli*. The human GM-CSF is readily isolated from the bacterial proteins because of its differential solubility and chromatographic properties. The bacterially synthesized form of the human GM-CSF contains an extra methionine residue at position 1, but otherwise it is identical to the polypeptide predicted from the cDNA sequence. The specific activity of 2.9×10^7 units/mg of protein for purified bacterially synthesized human GM-CSF indicates that despite the lack of glycosylation, the molecule is substantially in its native conformation. This molecule stimulated the same number and type of both seven- and 14-day human bone marrow colonies as the CSF α preparation from human placental conditioned medium. Human GM-CSF had no activity on murine bone marrow or murine leukemic cells. There was

no detectable, direct stimulation of adult human erythroid burst forming units (BFU-E) by the bacterially synthesized human GM-CSF. Although impure preparations containing native human GM-CSF (eg. human placental conditioned medium) stimulated the formation of mixed colonies, even in the presence of erythropoietin, the bacterially synthesized human GM-CSF failed to stimulate the formation of mixed colonies from adult human bone marrow cells. The bacterially synthesized human GM-CSF increased *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced superoxide production and lysozyme secretion. Antibody-dependent cytotoxicity and phagocytosis by human neutrophils was stimulated by the bacterially synthesized human GM-CSF and eosinophils were also activated in the antibody-dependent cytotoxicity assay.

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THE IDENTIFICATION of the cDNA clone for murine granulocyte-macrophage colony stimulating factor (mGM-CSF)¹ enabled the production of the biologically active polypeptide by COS cells^{1,2} and bacteria.^{3,4} The cDNA mGM-CSF also led to the isolation of full-length cDNAs for human GM-CSF⁵ (hGM-CSF) and confirmed the identity of the human CSF cloned from a COS cell expression library.⁶ The hGM-CSF expressed by COS cells⁷⁻⁹ appears to encode all of the in vitro biological activities associated with CSF α ¹⁰ prepared from human placental conditioned medium¹¹ and conditioned medium from the U5637 cell line.¹² The COS cell-derived hGM-CSF stimulated the proliferation and differentiation of granulocyte, macrophage, and eosinophil progenitor cells^{8,9} as well as the function of neutrophils.¹² Thus, the simian-derived carbohydrate moiety does not appear to alter the cellular specificity of the hGM-CSF.^{8,9} However, it is possible that a hGM-CSF polypeptide with a simian, hamster, or yeast carbohydrate moiety will be antigenic in vivo or that the different carbohydrate moiety will influence the in vivo distribution of the GM-CSF. We have produced hGM-CSF in bacteria and purified the polypeptide without any attached carbohydrate. These studies define the conditions necessary for the preparation and purification of bacterially synthesized hGM-CSF, the specific activity of the molecule, and its activities on progenitor as well as mature cells. In part, these results emphasize the continuing need to understand the interactions between subsets of human bone marrow cells.^{13,14}

MATERIALS AND METHODS

Preparation and Purification of Bacterially Synthesized Human GM-CSF

hGM-CSF cDNA was isolated from a library constructed using mRNA from the human monocytic cell line U937¹⁵ by screening candidate clones with a nick-translated probe derived from the coding sequences of mGM-CSF.³ The deduced amino acid sequence of the U937 hGM-CSF was identical to hGM-CSF produced by the leukemic cell line Mo(6) with the exception of the conservative

substitutions of a methionine by an isoleucine (amino acid 80 of the mature protein) and a threonine by an isoleucine (amino acid 100 of the mature protein). The amino acid for residue 100 of the mature protein deduced from a genomic clone was also isoleucine.¹⁶ These differences between cDNAs and the genomic sequences may represent allelic variations. Sequences coding for the mature hGM-CSF protein were placed in an *Escherichia coli* vector behind an inducible promoter as previously described for the mGM-CSF gene.³ The construction took advantage of the unique restriction endonuclease site (*Hga* I) located 58 nucleotides before the codon for the first amino acid of the mature protein (alanine). An oligonucleotide linker was synthesized to replace the deleted codons with the addition of an amino-terminal methionine codon to initiate translation in the prokaryote host. This linker contained substitutions of adenosine wherever retention of the native amino acid allowed.³ The noncoding 3' distal end of the cDNA was cleaved at the unique *Bal* I restriction endonuclease site and ligated to a similarly blunt-ended site (*Sma* I) in the expression vector. Once introduced into a suitable *E. coli* host (LON-, HPTR-) the induction of hGM-CSF production was initiated by temperature shift (28 °C to 48 °C). hGM-CSF accumulated over four hours to 8% to 10% of the total cell protein. Bacteria were harvested and GM-CSF extracted essentially as described for mGM-CSF.³ In induced cells hGM-CSF accumulated

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in dense bodies which were collected by centrifugation following breakage of the cells. After washing the pellet was resuspended in 6 mol/L guanidinium hydrochloride and 2 mmol/L β -mercaptoethanol and chromatographed over G-100 Sephadex in the same buffer. The highly purified hGM-CSF fractions were collected and the denaturant and reductant removed by slow dialysis against neutral Tris buffer. The final purification step was gradient elution from a reverse-phase support at pH 2.1 on an Ultrapore RPSC column (4.6×7.5 cm) from Beckman, Santa Clara, CA.¹⁷ The final purity of the hGM-CSF was assessed by sodium dodecyl sulfate (SDS) gel electrophoresis¹⁸ and automated gas phase amino acid sequence analysis.¹⁹ The protein concentration for the specific activity calculation was determined by amino acid analysis.²⁰

Assays on Bacterially Synthesized Human GM-CSF

The purified hGM-CSF preparation (approximately 0.3 mg/mL in acetonitrile [30%]: trifluoroacetic acid [0.1%]: water [70%]) was diluted 1:100 in 10% normal saline containing fetal calf serum (FCS) (10%, vol/vol) and 0.1 mL of the diluted sample (or serial dilutions thereof) was assayed in the 1-mL semisolid bone marrow cultures.

Human marrow cultures. Marrow and peripheral blood samples were obtained with the prior informed consent of patients according to a protocol approved by the Ethics Committee of the Royal Melbourne Hospital. Light density (ρ 1.070 g/cc) or nonadherent light density bone marrow cells (30,000) from patients with no hematological disease were cultured in 35-mm Petri dishes using 1 mL of Dulbecco's Modified Eagle's Medium and a final concentration of 20% FCS and 0.3% agar.²¹ Cultures were incubated for up to 14 days at 37 °C in a fully humidified atmosphere of 10% CO₂ in air. For erythroid colony formation, 1-mL cultures containing 30,000 light density (<1.070 g/cc) mononuclear or nonadherent mononuclear bone marrow cells in Iscove's Modified Dulbecco's Medium and a final concentration of 25% FCS and 0.3% agar were established. Cultures were incubated for 14 days in a fully humidified atmosphere of 5% CO₂ in air.

Colony formation (clones of more than 40 cells) was scored at 35 $\times$ magnifications using a dissection microscope after seven or 14 days of incubation. To determine the cellular composition of the resulting colonies, intact cultures were fixed using 2.5% glutaraldehyde and methanol and, after drying, were stained with Luxol Fast-Blue/hematoxylin. Colony classification was performed at 400 $\times$ magnifications.

Units of colony stimulating activity were calculated from the linear portion of the dose-response curve assigning 50 U/mL to the concentration stimulating the formation of 50% maximal colony numbers.

The human placental conditioned medium (HPCM) Stage II used in these experiments was prepared as described previously.¹⁰ The semipurified CSF α and β fractions were obtained using phenyl-Sepharose chromatography of medium conditioned by the human bladder cancer cell line U5637, using methods described previously.²²

Murine assays. Serial dilutions of bacterially synthesized hGM-CSF samples (0.1 mL) were added to agar cultures of 75,000 C57BL adult marrow cells in FCS (20%) or 20,000 12-day CBA fetal liver cells in cultures containing human plasma (20%). Control cultures contained 400 units of purified mGM-CSF,¹⁷ Multi-CSF,²³ G-CSF,²⁴ M-CSF,²⁵ or saline. Colony formation was scored after seven days of incubation. To determine whether bacterially synthesized hGM-CSF could initiate but not sustain colony formation by murine hematopoietic cells, 400 units of Multi-CSF were added after two days of incubation to replicate cultures of fetal liver cells and the cultures scored after a total incubation period of seven days. After

scoring, cultures were stained with Luxol Fast-Blue/hematoxylin and colonies classified according to cellular composition.

Microwell assays using the continuous hematopoietic cell lines 32D cl.3²⁶ (kindly supplied by Dr J. Greenberger, Boston) and FDC-P1²⁷ (kindly supplied by Dr T.M. Dexter, Manchester, England) were performed in microtiter trays using 200 cells per well in 10 μ L of Dulbecco's Modified Eagle's Medium with FCS (10%, vol/vol). Serial dilutions of 5 μ L of each test sample were added and direct cell counts performed after 48 hours of incubation.

To determine whether bacterially synthesized hGM-CSF was able to induce the differentiation of WEHI-3B(D⁺) cells,²⁸ serial dilutions of the hGM-CSF samples (0.1 mL) were added to agar cultures of 300 WEHI-3B(D⁺) cells. The percentage of colonies exhibiting differentiation was determined after seven days of incubation.²⁹ Control cultures contained 0.1 mL of serial dilutions of either serum from C57BL mice injected three hours previously with 5 μ g endotoxin³⁰ or semipurified human CSF β .¹⁰

Functional Activation of Mature Cells

Purification of human neutrophils and eosinophils. These cells were obtained from the peripheral blood of healthy volunteers after dextran sedimentation and centrifugation on a hypertonic gradient of Metrizamide (Nyegaard, Oslo) as previously described.³¹ The purity was higher than 95% for neutrophils and higher than 92% for eosinophils. The cells were resuspended in Eagle's Minimal Essential Medium supplemented with 10% FCS, 20 mmol/L HEPES buffer, and antibiotics.

Antibody-dependent cell mediated cytotoxicity assay. ADCC was performed as previously described.³² In brief, 40 μ L of ⁵¹Cr-labeled, trinitrophenyl (TNP)-coupled P815 cells (4×10^3) were incubated with 24 μ L of rabbit IgG anti-TNP (Rehovot, Israel), 80 μ L of purified human neutrophils or eosinophils (1.3×10^5) as effector cells, and 16 μ L of bacterially synthesized hGM-CSF for two and one half hours at 37 °C in V-bottom microtitre plates. Percent cytotoxicity was calculated from the formula: (test – control)/total – control $\times$ 100, where "control" was the ⁵¹Cr released by P815 cells in the presence of medium alone, and "total" was the ⁵¹Cr released by the addition of 5% Triton X-100.

Superoxide production. Purified neutrophils were incubated with medium or different concentrations of bacterially synthesized hGM-CSF for two hours at 37 °C. After this, 150 μ L of cells (10^6) were mixed with 100 μ L freshly prepared cytochrome-C (Sigma, St Louis, type VI, 12.4 mg/mL), 100 μ L N-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-6} mol/L) and made up to 1 mL with medium. The mixtures were then incubated at 37 °C for five minutes, after which the cells were rapidly cooled, centrifuged at 4 °C, and the supernatants transferred to plastic disposable cuvettes. Superoxide production was measured by the reduction of cytochrome-C³³ using an extinction coefficient of 21.1 mmol/L⁻¹.³⁴

The medium used throughout these assays was RPMI (free of phenol red) containing 2% FCS. In control experiments, bacterially synthesized hGM-CSF did not reduce cytochrome-C in the absence of cells, and superoxide dismutase (Sigma) completely abolished the reduction of cytochrome-C. The results are expressed as the means of triplicate determinations $\pm$ 1 standard deviation.

Phagocytosis assay. This assay measured the phagocytosis of serum-opsonized baker's yeast and was performed as described.⁹ In brief, 100 μ L purified neutrophils (2×10^7 /mL) were incubated with 250 μ L baker's yeast, 100 μ L diluted fresh autologous serum and 50 μ L bacterially synthesized hGM-CSF or medium with FCS for one hour at 37 °C. After this, the cells were centrifuged at 4 °C, resuspended in 50 μ L of cold PBS and smeared onto a slide. When eosinophils were tested, this assay was scaled down because of the relatively low numbers of eosinophils routinely obtained. The vol-

umes were 20 μ L of cells (10^7 /mL), 50 μ L of baker's yeast, 20 μ L of freshly obtained human serum and 10 μ L of bacterially synthesized hGM-CSF or medium. After incubation, cytochrome preparations were made. Slides were fixed in methanol and stained with Giemsa before being examined for the number of cells showing different numbers of phagocytosed yeast. The data are expressed as percentages after counting a minimum of 200 cells per slide.

Degranulation assay. This was carried out as previously described.³⁵ Briefly, purified neutrophils (5×10^6 /mL) pretreated with cytochalasin-B (Sigma) were incubated with different stimuli for 15 minutes at 37 °C. To these mixtures different concentrations of FMLP (Sigma) were added and the cells incubated a further 30 minutes at 37 °C. After incubation the cells were centrifuged and the released lysozyme assayed against *Micrococcus lysodeikticus* (Sigma). The optical density at 450 nm was measured at ten-second intervals for three minutes. Triplicate determinations (every ten seconds) were made and the amount of lysozyme released was calculated from a standard curve generated by egg white lysozyme (Sigma). Maximum values were obtained by using supernatants from neutrophils lysed with Triton X-100.

RESULTS AND DISCUSSION

Induction and Purification of Bacterially Synthesized Human GM-CSF

Recombinant hGM-CSF was produced in *E. coli* under the regulation of a heat-inducible promoter. After GM-CSF synthesis was induced by raising the culture temperature, the protein accumulated in aggregates as dense bodies within the cells. Following weakening of the cell walls by lysozyme treatment, cells were disrupted in a French pressure cell. The aggregates were collected by centrifugation and resuspended in 6 mol/L urea. hGM-CSF was initially purified from the solution by chromatography over Sephadex G100. Aliquots (2 to 5 mL) of the low-molecular weight fraction from the gel filtration containing GM-CSF were diluted with an equal volume of aqueous trifluoroacetic acid (0.15%, vol/vol) and loaded onto a reversed-phase Ultrapore RPSC column equilibrated with aqueous trifluoroacetic acid (0.15%, vol/vol). Several proteins were eluted from the column (Fig 1), but the earliest eluting material was not associated with biological activity. SDS-polyacrylamide gel analysis of the column fractions (Fig 2) indicated that the 16,000-dalton protein associated with the biologic activity was pure. Amino acid sequence analysis of 200 pmoles of the purified hGM-CSF

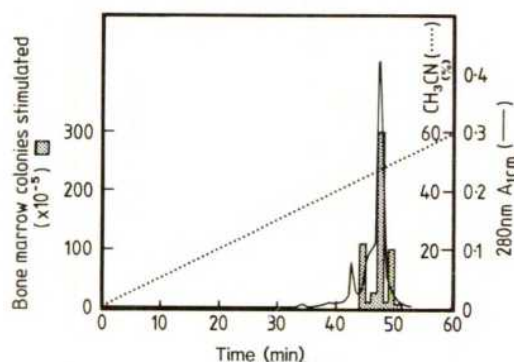


Fig 1. Reversed phase high-performance liquid chromatography purification of bacterially synthesized human GM-CSF.

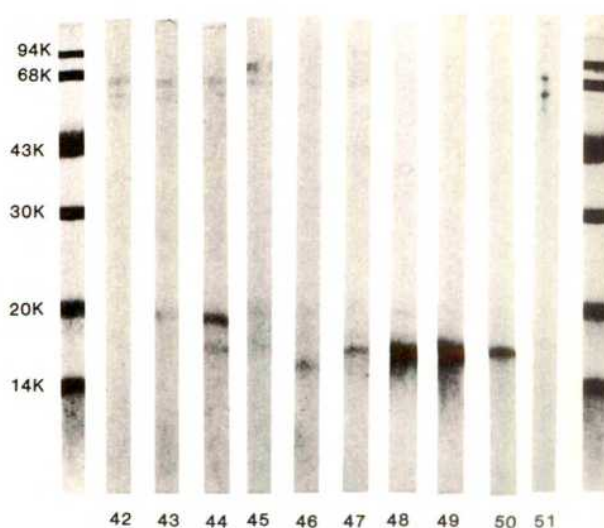


Fig 2. Electrophoretic analysis of purified bacterially synthesized human GM-CSF.

yielded a single amino acid at each cycle; the first residue detected was methionine, and the next 16 cycles yielded the sequence expected from the nucleotide sequence.⁶ Thus, this product is different from the natural molecule in that not only is the carbohydrate moiety missing, but there is also an *N*-terminal methionine which is not removed during synthesis. The results of the quantitative amino acid analysis of the purified bacterially synthesized hGM-CSF were in excellent agreement with the composition predicted from the nucleotide sequence. The optical density at pH 2.1 in the presence of acetonitrile (30%, vol/vol), together with the amino acid analysis yielded a molar extinction coefficient of 2×10^4 moles⁻¹ cm⁻¹. Attempts to determine a precise specific activity for the purified bacterially synthesized hGM-CSF are complicated by the wide variation in sensitivity of different bone marrow samples and variations in the frequency of colony-forming cells for different human bone marrow samples. The standard assays were performed using between 30,000 and 50,000 bone marrow cells per milliliter. Eight separate bone marrows stimulated by the purified bacterially synthesized hGM-CSF yielded an average specific activity of $2.9 \pm 1.9 \times 10^7$ U/mg of protein after seven days in culture. The same four samples yielded a specific activity of $2.9 \pm 0.7 \times 10^7$ U/mg of protein after 14 days in culture. The ratio of day-7 to day-14 colonies was dependent on the bone marrow sample and varied from 0.3 to 1.6. In general, impure samples of hGM-CSF (eg, human placental conditioned medium or bladder carcinoma conditioned medium) contained a mixture of both hGM-CSF (CSF α) and hGM-CSF (CSF β). The CSF β preparation preferentially stimulates colony formation at day 7; thus, the ratio of the number of colonies at day 7 to day 14 (approximately three) tends to be considerably higher than for GM-CSF and the CSF α preparation. Similarly, a mixture of the bacterially synthesized GM-CSF and the CSF β preparation generally stimulates more day-7 colonies than either stimulus by itself. Each molecule preferentially stimulates a defined subset of GM-progenitor cells. After 14 days in culture the bacterially

synthesized hGM-CSF stimulated as many colonies as preparations of CSF α ¹⁰ from human placental conditioned media.¹¹

Our method of preparation and purification indicates that the bacterially synthesized hGM-CSF is stable in 6 mol/L urea, as well as at pH 2.1 in the presence of 40% acetonitrile. Indeed, at low pH the bacterially synthesized hGM-CSF appears to retain its full biologic activity indefinitely. However, initial attempts to store this hGM-CSF at neutral pH in the absence of urea have led to precipitation of the molecule.

Concentration Dependence of Colony Formation

The purified hGM-CSF was titrated and assayed on eight separate bone marrow aspirates: both the seven-day (Fig 3A) and 14-day (Fig 3B) cultures indicated that maximal stimulation of colony formation was achieved at concentrations of 500 pmol/L or greater. Half-maximal stimulation occurred between 60 pmol/L and 120 pmol/L and the minimum detectable hGM-CSF concentration using the semisolid agar assay was 10 pmol/L for both time points.

The direct action of bacterially synthesized hGM-CSF on human colony forming cells was examined by transfer of day-5 clones to plates containing the bacterially synthesized hGM-CSF, human placental conditioned medium, or saline (Fig 4). The bacterially synthesized hGM-CSF continued to stimulate colony growth and GM-CSF is undoubtedly one of the active hematopoietic growth factors in the HPCM.

Morphology of Colonies

In two separate experiments, the types of colonies stimulated by the bacterially synthesized hGM-CSF have been compared to the colony types obtained with unfractionated HPCM, CSF α , and CSF β (Table 1). The bacterially synthesized hGM-CSF stimulated pure neutrophil (G), macrophage (M) and eosinophil (Eo) colonies as well as mixed granulocyte-macrophage colonies. At day 7 all preparations of CSF stimulated mainly granulocytic colonies. However, the bacterially synthesized hGM-CSF and CSF α also stimu-

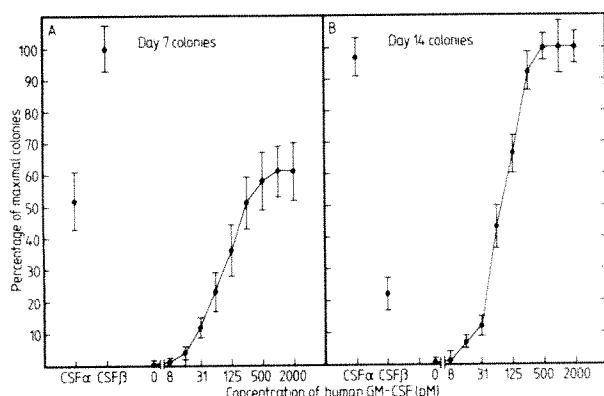


Fig 3. Effect of GM-CSF on colony growth for eight normal human bone marrow samples. (A) Day 7; (B) Day 14. The results are presented as the mean and standard deviations. The results of assays with optimal concentrations of CSF α and CSF β prepared from human placental conditioned medium are also displayed.

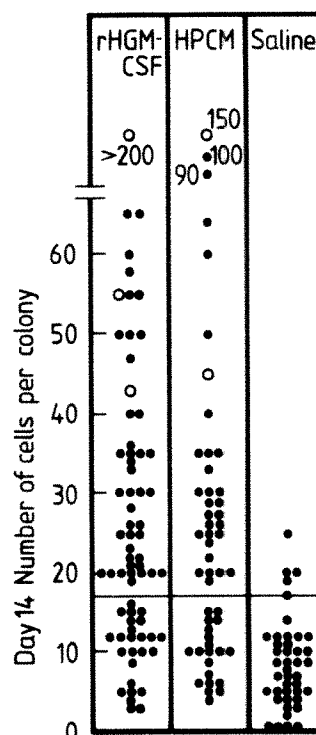


Fig 4. Growth of colonies after transfer of Day 5 human bone marrow colonies to recipient plates with either bacterially synthesized hGM-CSF, human placental conditioned medium (HPCM), or saline. The solid circles represent colonies composed of neutrophils, macrophages, or both, and the open circles represent the eosinophil colonies.

lated a small proportion of Eo colonies. As expected, for both HPCM and CSF α the proportions of pure M and pure Eo colonies were increased at 14 days. Thus, the bacterially synthesized hGM-CSF stimulated a similar range of colonies to the CSF α .

The bacterially synthesized hGM-CSF was used in an attempt to stimulate the hematopoietic progenitor cells in murine bone marrow. Even at a concentration of 2 nmol/L no colonies or clusters were observed after seven days in culture. The hGM-CSF did not enhance the survival of multi-CSF-responsive progenitor cells in the delayed addition assay. Similarly, there was no effect of this hGM-CSF on several murine cell lines which were known to respond to murine hematopoietic growth factors: WEHI-3BD⁺ (G-CSF-responsive) did not differentiate in response to hGM-CSF, FDCP1 (GM-CSF- or Multi-CSF-dependent) and 32D cells (Multi-CSF-dependent) did not proliferate in the presence of hGM-CSF (data not shown).

Effect of Bacterially Synthesized Human GM-CSF on Erythroid Progenitor Cells

The colony types stimulated by human tissue-derived conditioned media (eg, human placental conditioned medium, [HPCM]) have been studied extensively using a range of partially purified hematopoietic progenitor cells.^{36,37} Studies by Sieff et al with a hGM-CSF partially purified from transfected COS cell conditioned medium indicated

Table 1. Morphology of Human Bone Marrow Colonies Stimulated by Bacterially Synthesized hGM-CSF

Incubation Time	Experiment Number	Stimulus	Mean Number of Colonies*	Colony Type (%)			
				G	GM	M	Eos
7 days	1	Saline	0	—	—	—	—
		HPCM	275	84	0	16	0
		hGM-CSF (recombinant)	230	83	0	11	6
		CSF α	232	83	0	17	0
		CSF β	278	82	0	18	0
	2	Saline	0	—	—	—	—
		HPCM	146	64	2	34	0
		hGM-CSF (recombinant)	98	58	3	36	3
		CSF α	98	56	8	32	4
		CSF β	149	64	5	31	0
14 days	1	Saline	0	—	—	—	—
		HPCM	98	42	6	39	13
		hGM-CSF (recombinant)	95	40	3	42	15
		CSF α	93	52	1	38	9
		CSF β	31	72	0	28	0
	2	Saline	3	0	0	100	0
		HPCM	87	31	13	50	6
		hGM-CSF (recombinant)	89	23	12	52	13
		CSF α	85	24	13	49	14
		CSF β	30	67	12	21	0

* 3×10^4 light density nonadherent human bone marrow cells were cultured in 1 mL of semisolid agar and examined after seven and 14 days. The agar cultures were fixed and stained and the colonies analyzed to determine the proportion of G, neutrophil; M, macrophage; GM, neutrophil-macrophage and EOS, eosinophil colonies.

some potentiating effect of the GM-CSF on BFU-E proliferation.³⁸ Similarly, their results with highly purified fetal BFU-E suggested that pure hGM-CSF stimulates the proliferation and differentiation of these cells. However, the initial results with purified mGM-CSF on murine fetal liver progenitor cells³⁹ indicated that only a subset of the fetal BFU-E appeared to be responsive to GM-CSF and the proportion of adult BFU-E responsive to GM-CSF appears to be quite small.

In a report of experiments with the COS cell-derived recombinant hGM-CSF and human fetal progenitor cells,⁴⁰ it was noted that even though the BFU-E represented 30% of the cell population when the culture cell density was below 100 cells/mL no colonies developed in the presence of GM-CSF. It is possible that the GM-CSF stimulated accessory cells to produce BFU-E stimulating factors. Initially, we attempted to detect the effect of the bacterially synthesized GM-CSF on BFU-E in mononuclear and nonadherent mononuclear cells from human bone marrow cells (Tables 2 and 3).

The light density mononuclear cells from human bone marrow did not produce erythroid colonies in the absence of erythropoietin (Table 2). In the presence of erythropoietin, erythroid colonies developed. Addition of a source of CSF (HPCM) did not change the frequency of erythroid colonies. However, the neutrophil-, eosinophil-, and macrophage-containing colonies appeared as expected. Identical effects were observed with the bacterially synthesized human GM-CSF (at both 2.5 nmol/L and 50 nmol/L). When the addition of erythropoietin was delayed for three to four days, there was a slight decrease in erythroid colony numbers but neither HPCM nor the bacterially synthesized hGM-CSF influenced the survival of these erythroid progenitors.

Table 2. No Effect of Bacterially Synthesized hGM-CSF on Erythroid Colony Formation From Mononuclear Human Bone Marrow Cells

Stimulus*	Day of Epo† Addition	Number of Colonies‡		
		Pure Erythroid	Mixed Erythroid	Non-erythroid
—	—	0	0	0
—	0	24 ± 5	0	16 ± 3
HPCM	0	25 ± 2	0	98 ± 5
HPCM	—	0	0	110 ± 3
hGM-CSF (2.5 nmol/L)	—	0	0	11 ± 12
hGM-CSF (2.5 nmol/L)	0	20 ± 8	0	102 ± 4
hGM-CSF (50 nmol/L)	—	0	0	124 ± 11
hGM-CSF (50 nmol/L)	0	13 ± 5	0	104 ± 11
—	3	17 ± 6	0	16 ± 5
—	4	13 ± 5	0	17 ± 4
HPCM	3	16 ± 1	0	117 ± 5
HPCM	4	15 ± 6	0	121 ± 13
hGM-CSF (2.5 nmol/L)	3	21 ± 8	0	87 ± 9
hGM-CSF (2.5 nmol/L)	4	21 ± 4	0	65 ± 10
hGM-CSF (50 nmol/L)	3	14 ± 2	0	99 ± 9
hGM-CSF (50 nmol/L)	4	12 ± 1	0	104 ± 15

—, Epo not added.

*The stimulus HPCM (0.1 mL) or the diluted, purified bacterially synthesized hGM-CSF (0.1 mL) was added at the start of the 14-day cultures of 3×10^4 light density bone marrow cells in the standard soft agar assay.²¹

†Partially purified erythropoietin (Epo) was added to a final concentration of 1.5 U/mL either at the start of the cultures (day 0) or at three or four days after initiating the assay.

‡For all assays three replicate plates were scored after 14 days. The table entries represent the mean ± SD.

Table 3. No Effect of Bacterially Synthesized hGM-CSF on Erythroid Colony Formation from Nonadherent Mononuclear Human Bone Marrow Cells*

Stimulus	Day of Epo Addition	Number of Colonies		
		Pure Erythroid	Mixed Erythroid	Non-erythroid
—	—	0	0	14 ± 2
—	0	38 ± 4	0	13 ± 3
HPCM	0	62 ± 4	1 ± 1	140 ± 11
HPCM	—	0	0	151 ± 10
hGM-CSF (2.5 nmol/L)	—	0	0	97 ± 6
hGM-CSF (2.5 nmol/L)	0	22 ± 1	0	97 ± 1
hGM-CSF (50 nmol/L)	—	0	0	227 ± 6
hGM-CSF (50 nmol/L)	0	26 ± 1	0	217 ± 5
—	3	12 ± 1	0	20 ± 6
—	4	8 ± 2	0	17 ± 7
HPCM	3	31 ± 7	1 ± 1	95 ± 2
HPCM	4	31 ± 5	0	90 ± 3
hGM-CSF (2.5 nmol/L)	3	11 ± 2	0	104 ± 6
hGM-CSF (2.5 nmol/L)	4	9 ± 2	0	105 ± 6
hGM-CSF (50 nmol/L)	3	16 ± 1	0	191 ± 2
hGM-CSF (50 nmol/L)	4	4 ± 1	0	206 ± 11

*See footnote to Table 2 for technical details of these experiments.

After removing the adherent cells, HPCM but not the bacterially synthesized hGM-CSF increased the cloning efficiency of the erythroid progenitor cells (Table 3). When the adherent cells are removed, a subset of the erythroid progenitors appears to be stimulated by HPCM. Even when the erythropoietin is added three or four days after the initiation of the cultures with HPCM this synergistic effect of HPCM can be observed (Table 3). Again, approximately half the erythroid colonies appear to be stimulated by a molecule present in human placental conditioned medium. It is unlikely that GM-CSF is related to this phenomenon, as the bacterially synthesized hGM-CSF did not modulate the number of 14-day erythroid colonies.

When both erythropoietin and hGM-CSF were present, their effects appeared to be independent. No extra erythroid colonies were observed compared to the cultures stimulated with erythropoietin alone. In contrast, unfractionated HPCM consistently increased (two- to fourfold) the number of erythroid colonies developing from the nonadherent light density mononuclear bone marrow cells. Mixed erythroid colonies⁴¹ were only observed in the presence of the unfractionated HPCM. Neither the bacterially synthesized GM-CSF, the erythropoietin, nor the mixture of both were able to stimulate human mixed colonies. At concentrations greater than 500 pmol/L, the bacterially synthesized GM-CSF stimulated the same range of nonerythroid colonies as the optimum concentration of human placental conditioned medium.

The lack of erythroid (BFU-E) potentiation by hGM-CSF could be due to the presence of accessory cells which suppress the GM-CSF-responsive BFU-E. However, the previously reported effects of GM-CSF may also be indirect (ie, via a GM-CSF-responsive cell which produces an erythroid factor). Understanding the interactions between the different cells controlling human erythropoiesis will require a detailed analysis of the proliferation of single cells and subsequent cell-mixing experiments.

Action of Bacterially Synthesized hGM-CSF on Mature Hematopoietic Cells

As well as stimulating the proliferation of the myeloid progenitor cells, the hGM-CSF purified from the Mo cell line is able to stimulate some of the functions of mature neutrophils^{8,9,12} and eosinophils.²⁰ We have measured the effect of the bacterially synthesized GM-CSF on four parameters of granulocyte function: superoxide (O_2^-) production,^{33,34} phagocytosis,⁹ lysozyme secretion,³⁵ and antibody-dependent cell-mediated cytotoxicity.³² At concentrations of hGM-CSF above 10 pmol/L both purified neutrophils and eosinophils were stimulated maximally to kill tumor target cells, and stimulation of killing was still detectable at 1 pmol/L (Fig 5). The concentration dependence of neutrophil and eosinophil activation was very similar (Fig 5). A similar concentration-dependent effect on neutrophil function was obtained by Weisbart et al⁸ for the inhibition of neutrophil migration using either recombinant hGM-CSF produced by transfected monkey COS cells or the hGM-CSF purified from the Mo cell line. Thus, our results show that hGM-CSF does not require carbohydrate moieties for its action in vitro.

COS cell-derived hGM-CSF has been previously found to stimulate phagocytosis.⁹ A comparison of the effects of *E. coli*-synthesized hGM-CSF on neutrophil phagocytosis of yeast organisms in the presence or absence of serum is displayed in Fig 6. In the presence of fresh human serum, the bacterially synthesized hGM-CSF (4 nmol/L) activated large numbers of neutrophils to phagocytose several yeast particles (Fig 6). Heating of human serum for 30 minutes at 56 °C abrogated the GM-CSF stimulation of neutrophils to phagocytose heat-killed yeast (data not shown), suggesting that complement is the opsonic factor present in human serum, and that the stimulation by hGM-CSF is dependent on the presence of this opsonin. There was a 50% increase in the number of neutrophils which phagocytosed the heat-killed yeast (Fig 6).

The response of neutrophils to bacterial products also appears to be stimulated by hGM-CSF. The secretion of lysozyme by neutrophils was dependent on the presence of chemotactic agents (Table 4). In the absence of a chemotactic peptide such as FMLP,⁴² there was no activation of lysozyme secretion. However, preincubation of neutrophils with the bacterially synthesized hGM-CSF enhanced the FMLP-induced release of lysozyme from purified neutrophils by 60% (Table 4). At low concentrations of the chemotactic peptide (10^{-8} mol/L) the hGM-CSF was more

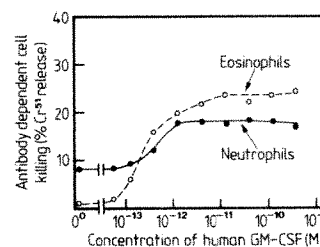


Fig 5. Effect of GM-CSF concentration on the ability of human neutrophils or eosinophils to kill antibody-coated P815 mastocytoma cells.

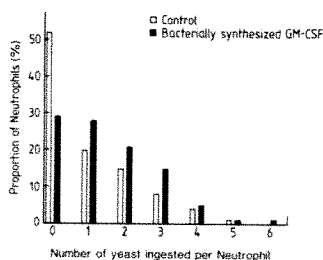


Fig 6. Effect of GM-CSF (4 nmol/L) on the ability of human neutrophils to phagocytose yeast.

effective than lipopolysaccharide.⁴³ Bacterially synthesized hGM-CSF also enhanced FMLP-mediated stimulation of superoxide production by human neutrophils (Fig 7). In the absence of hGM-CSF and FMLP, superoxide production by human neutrophils was not detectable. The chemotactic peptide by itself induced O_2^- production to 1.3 nmoles/min/ 10^6 cells. In the absence of FMLP, hGM-CSF had little or no effect on O_2^- production (Fig 7). However, preincubation of neutrophils with GM-CSF followed by FMLP increased the amount of O_2^- production sevenfold over the level induced by FMLP alone. The interaction between FMLP and GM-CSF was concentration dependent: the maximal rate of O_2^- production was achieved at 200 pmol/L, but the synergism was still evident when the hGM-CSF concentration was 10 pmol/L (Fig 7).

Thus, GM-CSF is capable of enhancing at least three separate systems in human neutrophils: the release of both lysozyme and O_2^- (upon stimulation with FMLP), phagocytosis of yeast (complement-mediated), and killing of tumor target cells (antibody-dependent).⁴⁴ In its ability to activate mature neutrophils, this bacterially-synthesized hGM-CSF behaves very similarly to the recombinant hGM-CSF produced by transfected monkey COS cells (ref 9, and Lopez et al, in preparation).

We have demonstrated the action of purified, bacterially synthesized hGM-CSF on a range of hematopoietic progenitor and mature cells. This GM-CSF has a specific activity in the same range as the recombinant GM-CSF purified from medium conditioned by Mo cells³⁸ or the medium from COS cells transfected with cDNA for hGM-CSF¹² (Table 5). Thus, neither the addition of the extra *N*-terminal methionine residue, nor the absence of carbohydrate appear to affect the in vitro activity of hGM-CSF. The detailed morphological distribution of the colonies stimulated by both the *E. coli*-synthesized and COS cell-stimulated hGM-CSF

Table 4. Effect of Bacterially Synthesized hGM-CSF on Lysozyme Release by Neutrophils

Degranulating Stimulus	Lysozyme Secretion* Following Preincubation with hGM-CSF (pmol/L)†			
	Nil	30	120	300
Nil	0 ± 1	0 ± 0	0 ± 0	0 ± 1
FMLP 10 ⁻⁷ mol/L	27 ± 4	35 ± 1	70 ± 2	66 ± 5
FMLP 10 ⁻⁸ mol/L	106 ± 6	122 ± 1	147 ± 8	150 ± 8

*Lysozyme secretion expressed as the average U/mL/min/ 10^6 neutrophils ± SD (for three replicate cultures).

†Final concentration of bacterially synthesized hGM-CSF.

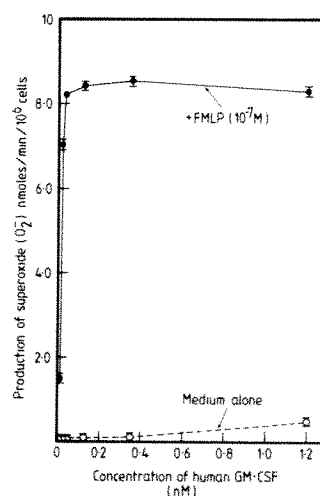


Fig 7. Enhancement by human GM-CSF of FMLP-induced superoxide production by human neutrophils.

varies considerably and is dependent on the specific bone marrow being assayed. However, both forms stimulate predominantly neutrophilic granulocyte colonies at day 7 (Table 5), and by day 14 most of the colonies contain macrophages. There is a similar increase in the proportion of eosinophil colonies with both GM-CSFs by day 14 (Table 5).

Our attempts to show the direct effects of hGM-CSF on erythroid progenitor cells (ie, BFU-E) have been unsuccessful. Despite the availability of a pure stimulus such as the bacterially synthesized hGM-CSF, the purity of our hematopoietic progenitor cells has been insufficient to prove that the hGM-CSF acts directly on progenitors of the erythroid series. At least one study using COS cell-derived recombi-

Table 5. Comparison of *E. coli*-Derived and COS Cell-Derived hGM-CSF

Property	hGM-CSF					
	<i>E. coli</i> -Derived			COS Cell-Derived		
Apparent molecular weights	16,000			19,000–22,000		
Specific Activity (U/mg/ 10 ⁴ bone marrow cells)	1 × 10 ⁷			1 × 10 ⁷ –5 × 10 ⁷		
Morphology of myeloid colonies	(%)			(%) ⁴		
		Day 7	Day 14		Day 8	Day 14
	G	70	31	G	70	8
	GM	4	8	GM	20	16
	M	24	47	M	5	51
	Eo	2	14	Eo	5	25
BFU-E Responsiveness	No effect			(a) Stimulated “directly” (b) No effect (c) Inhibitory in the presence of erythroid potentiating activity		
Responsive mature cells	G, Eo			G, Eo, and monocytes ⁴⁶		

Abbreviations: G, granulocytes; GM, granulocyte-macrophages; M, monocytes; Eo, eosinophils.

nant hGM-CSF also reported that there was no effect on BFU-E, although in the presence of erythroid potentiating activity and erythropoietin, the human GM-CSF appeared to inhibit BFU-E.⁴⁵ Others appear to have shown effects of COS cell-derived hGM-CSF on highly purified BFU-E.⁴⁰ It will be necessary to study the effects of pure hGM-CSF on single BFU-E before we can be sure that the hGM-CSF has a direct effect on erythropoiesis.

The availability of *E. coli*-synthesized hGM-CSF with essentially identical biologic properties to natural hGM-CSF will provide sufficient quantities of this molecule for clinical testing. The absence of any foreign carbohydrate moiety should reduce problems with immunologic neutralization

during clinical applications. The absence of carbohydrate side chains has allowed the development of monoclonal antibody reagents which can distinguish between the native hGM-CSF (as is present in human placental conditioned medium) and the bacterially synthesized GM-CSF. Thus, it will be possible to monitor the serum levels of the administered GM-CSF during patient treatment. The bacterially synthesized hGM-CSF should be suitable both for studies aimed at increasing the rate of recovery of myeloid cells after cytotoxic treatment and for attempts to activate neutrophils and/or eosinophils in patients with poor neutrophil function, eg, older patients, immunosuppressed patients or patients with chronic infections.

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New Abnormalities in the Morphology, Cell Surface Receptors, and Electrolyte Metabolism of In(Lu) Erythrocytes

By Mark M. Udden, Masato Umeda, Yukiko Hirano, and Donald M. Marcus

The In(Lu) phenotype is inherited as an autosomal dominant trait and is characterized by suppression of the Lutheran, P₁, i, and Au^a erythrocyte blood group antigens. We have developed a monoclonal antibody (L21) that strongly agglutinates all erythrocytes except In(Lu), and we have identified eight In(Lu) individuals among 42,000 blood donors tested. Studies of two families confirmed the dominant mode of inheritance and revealed several new features of this phenotype. The erythrocytes of all five affected individuals from the two families exhibited diminished hemagglutination by the lectin concanavalin A, although they reacted normally with several other lectins. The erythrocytes of two affected individuals in one family exhibited marked acanthocytosis. The erythrocytes of the *proposita* of the other family exhibited a mild degree of

poikilocytosis, but the cells of the other two affected individuals in this family had normal morphology. The osmotic fragility of fresh In(Lu) erythrocytes was normal, but after incubation for 24 hours at 37 °C in plasma the In(Lu) cells exhibited a marked increase in resistance to osmotic lysis. During the incubation period the erythrocytes lost K⁺ and their total cation content was diminished. These data indicate that in addition to the suppression of blood group antigens noted previously, the In(Lu) phenotype includes a variety of morphological abnormalities and a defect in electrolyte metabolism. The use of L21 and similar monoclonal antibodies provides a more sensitive means of detecting In(Lu) erythrocytes than typing with human anti-Lu^b antisera.

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THE MAJOR ANTIGENS of the Lutheran blood group system are the codominant antigens Lu^a and Lu^b.¹ Two rare Lu(a-b-) phenotypes have been described. One is inherited as an autosomal recessive trait; these erythrocytes contain no detectable Lu^a or Lu^b antigens and the expression of other blood group antigens is normal.¹⁻⁵ The other Lu(a-b-) phenotype, In(Lu), exhibits several exceptional features: it is inherited as an autosomal dominant, the expression of antigens from other blood group systems (P₁, i, and Au^a) is suppressed, and weak expression of the Lu^a and Lu^b antigens can be detected by adsorption and elution of anti-Lu^a and anti-Lu^b from erythrocytes.^{1,4-7} No information is available about the biochemical nature of the Lu^a and Lu^b antigenic determinants.

Recently several monoclonal antibodies have been described that strongly agglutinate all erythrocytes except In(Lu) cells, which give weak or negative reactions; recessive Lu(a-b-) cells react normally. The H86 monoclonal antibody (H86 mAb) described by Knowles et al⁸ was elicited by immunization of mice with the human T cell leukemia line HSB-2, and A3D8 mAb was raised by immunization with Sezary T cells.^{9,10} Both antibodies bind to most human blood mononuclear cells, and A3D8 immunoprecipitates 80-kD

and 170-kD proteins from erythrocytes. In(Lu) erythrocytes contain only traces of immunoprecipitable 80-kD protein but the 170-kD protein appears to be present in increased abundance.¹⁰

We have produced a monoclonal antibody (L21) that also reacts very weakly with In(Lu) cells and we have used this antibody to identify individuals with the In(Lu) phenotype. We describe here newly identified morphologic and functional abnormalities of In(Lu) erythrocytes.

MATERIALS AND METHODS

Erythrocytes. Fresh samples of venous blood were obtained in heparinized tubes and stored at 4 °C in Alsever's solution.

Antibodies and lectins. Monoclonal antibody L21 (IgG₁, κ) was produced by intravenous immunization of BALB/c mice with pooled umbilical cord red cells, and it was obtained in the same fusion that yielded five monoclonal antibodies against glycophorin A. Details of the fusion and cloning procedures were published previously.¹¹ When tested against a large panel of erythrocytes in two blood group reference laboratories, Ortho Diagnostic Systems, Raritan, NJ, and Gamma Biologicals, Houston, TX, L21 strongly agglutinated all erythrocytes except those of In(Lu) phenotype. Monoclonal antibody A3D8⁹ was kindly provided by Drs M. Telen and B. Haynes, and H86⁸ was a gift from Dr R. Knowles. Concanavalin A (Con A), *Lens culinaris*, *Ricinus communis*, and *Arachis hypogaeae* were purchased from Vector Laboratories, Burlingame, CA, and E-Y Laboratories, San Mateo, CA.

Immunological tests. Hemagglutination tests were performed by incubating 50 μL of serial twofold dilutions of monoclonal antibodies or lectins with 50 μL of a 1% erythrocyte suspension for one hour at room temperature in a 96-well microtiter plate (Dynatech Laboratories, Alexandria, VA). L21 was labeled with ¹²⁵I by the iodogen procedure¹² and the radiolabeled antibody was separated from free ¹²⁵I by gel filtration on a PD-10 column (Pharmacia Fine Chemicals, Piscataway, NJ).

Binding of radiolabeled antibodies to erythrocytes. Erythrocytes were washed three times with phosphate-buffered saline (150 mmol/L NaCl, 20 mmol/L PO₄, pH 7.4, PBS) and were suspended in PBS containing 10 mg/mL bovine serum albumin (1% BSA-PBS). 2 × 10⁸ erythrocytes in 0.5 mL 1% BSA-PBS were incubated with ¹²⁵I-labeled L21 for one hour at room temperature with occasional shaking, washed three times with 1% BSA-PBS, and counted in a gamma counter (LKB, Bromma, Sweden). For the competitive binding assay, ¹²⁵I-labeled L21 was mixed with varying

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concentrations of A3D8 or H86 and the binding of ^{125}I -labeled L clone to erythrocytes was measured.

Erythrocyte morphology and osmotic fragility. Freshly drawn blood anticoagulated with EDTA was used for preparation of Wright's-stained peripheral blood films. Erythrocytes were prepared for scanning electron microscopy by washing three times with 150 mmol/L NaCl containing 5 mmol/L Tris, pH 7.4, and then fixed in buffer containing 3% glutaraldehyde. The fixed erythrocytes were attached to polylysine hydrobromide-coated glass coverslips and then dehydrated by immersion in a series of acetone solutions increasing in concentration from 50% to 100% acetone. They were then critical point-dried from acetone and liquid CO_2 , sputter-coated with gold, and examined in a JEM 100C Teuscan electron microscope (JEOL, Peabody, MA) in the secondary electron imaging mode at 20 kilo-electron volts. Erythrocyte osmotic fragility was determined on fresh and incubated whole blood anticoagulated with heparin as described by Beutler.¹³ Erythrocyte Na^+ and K^+ concentrations were determined by atomic absorption spectroscopy in the emission mode using an atomic absorption spectrometer (Perkin-Elmer, Norwalk, CT). Measurements were made on fresh and incubated erythrocytes washed four times with isotonic MgCl_2 and then lysed in 0.1% LiCl. Other hematologic measurements, hemoglobin, WBC, and RBC counts were determined using a Baker Hematology Series 150 cell counter (Baker, Allentown, PA).

RESULTS

Expression of antigens and lectin receptors. Erythrocytes of types Lu(a+b+), Lu(a-b+), Lu(a+b-) and Lu(a-b-) were agglutinated by L21 at dilutions of 1:6,000 to 1:25,000. When the antibody, diluted 1:2,000, was used to test 42,000 blood donors, eight individuals were found whose red cells gave weak or negative reactions.¹⁴ Two In(Lu) donors and their relatives, family C and family S (Fig 1) were selected for further investigation. Erythrocytes from 11 donors from the two families were tested for hemagglutination by L21 and A3D8 (Table 1). Erythrocytes of the two

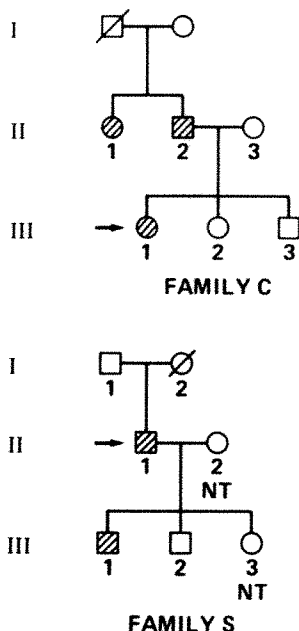


Fig 1. Pedigrees of two In(Lu) families. NT, not tested; $\square$, $\square$, In(Lu); $\square$, $\square$, normal; and $\square$, $\square$, deceased.

Table 1. Agglutination of Erythrocytes by Monoclonal Antibodies

Family C	L21*	A3D8*
I-2	20,480 (++++)†	1,280 (+++)
II-1	640 (++)	160 (+)
II-2	640 (+)	80 (+)
II-3	20,480 (++++)	640 (++++)
III-1	640 (+)	80 (+)
III-2	20,480 (++++)	640 (++++)
III-3	20,480 (++++)	640 (++++)
Family S	L21*	A3D8‡
I-1	25,600 (++++)	6,400 (++++)
II-1	ND§	ND
III-2	25,600 (++++)	6,400 (++++)
III-1	ND	ND

*The monoclonal antibodies were partially purified by precipitation with ammonium sulfate.

†Strength of hemagglutination was graded as follows: +++, all cells were agglutinated in large aggregates; ++, small aggregates; +, very small aggregates.

‡Ascites.

§Hemagglutination was not detected.

members of family S (II-1 and III-1) failed to react with both mAbs, and three members of family C (II-1, II-2 and III-1) gave weak agglutination with L21 and A3D8.

The direct binding of L21 to erythrocytes was measured by using radiolabeled L21 mAb. Erythrocytes from all five donors of the In(Lu) phenotype exhibited marked reduction of binding by L21 ($21.1 \pm 6.5\%$ of normal). Figure 2 shows typical binding curves obtained with erythrocytes from family C members. Two individuals with the In(Lu) phenotype (II-1 and II-2) showed marked reduction of binding compared to unaffected family members. Less suppression of binding (34% of normal) was observed with III-1 of family C (data not shown). The extent of the reduction in binding is consistent with the results obtained with the hemagglutination assays shown in Table 1, and the data suggest incomplete and variable suppression of the antigen by the In(Lu) gene.

The In(Lu) gene also inhibits the expression of the P_1 and i

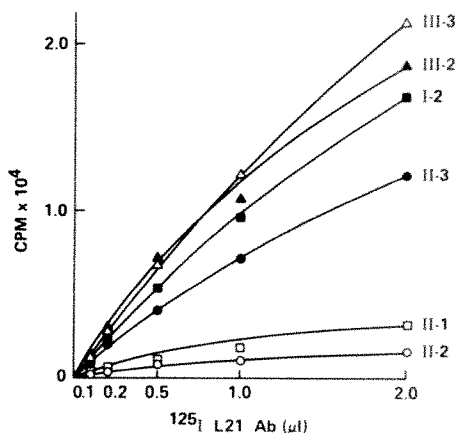


Fig 2. Binding of ^{125}I -labeled L21 monoclonal antibody to erythrocytes of the C family. The erythrocytes of the probanda, III-1, were not tested on this occasion.

Table 2. Agglutination of Erythrocytes by Concanavalin A

	Con A concentration ($\mu\text{g/mL}$)					
	62	31	16	8	4	2
Family C						
I-2	+++	+++	+++	+++	++	-
II-1 [In(Lu)]	++	++	++	+	-	-
II-2 [In(Lu)]	++	++	++	+	-	-
II-3	++	+++	+++	+++	++	-
III-1 [In(Lu)]	++	++	+	-	-	-
III-2	+++	+++	+++	+++	++	-
III-3	+++	+++	+++	+++	++	-
Family S						
I-1	+++	+++	+++	+++	-	-
II-1 [In(Lu)]	++	++	+	+	-	-
III-1 [In(Lu)]	++	++	+	-	-	-
III-2	+++	+++	+++	++	-	-

antigens, which are known to be carbohydrate structures.¹⁵⁻¹⁷ In order to study the effect of the In(Lu) gene on other carbohydrate structures, hemagglutination by several lectins was examined. All five In(Lu) members of both families exhibited reduced hemagglutination by concanavalin A (Table 2). Hemagglutination by other lectins (*Lens culinaris*, *Ricinus communis*, and *Arachis hypogaeae*), was normal (data not shown).

Erythrocyte morphology. In family S the In(Lu) phenotype was associated with acanthocytic erythrocytes as shown in Fig 3. The Wright's-stained peripheral blood films of In(Lu) individuals II-1 and III-1 (Figures 3A, 3C) show numerous acanthocytes whereas the unaffected family members had the expected discocyte morphology of normal erythrocytes. The In(Lu) individuals had other poikilocytes, but there were no Howell-Jolly bodies, targets, or siderocytes present to suggest an asplenic blood picture. Acanthocytes and other bizarre erythrocyte shapes were also observed when In(Lu) cells were washed free of plasma, fixed with glutaraldehyde and then viewed by scanning electron microscopy (Fig 4). The erythrocyte morphology of the members of family C was normal except for the proposita, whose cells demonstrated a mild degree of poikilocytosis and the pres-

ence of an occasional acanthocyte. Other common hematologic tests, including reticulocyte count, white blood cell count, packed cell volume, hemoglobin, red cell count, and the derived red blood cell indices were within normal limits in two In(Lu) individuals (family C, III-1 and family S, II-1). The absence of anemia and reticulocytosis suggests that the In(Lu) phenotype is not associated with significant hemolysis. The In(Lu) probands are healthy and have no past history or physical findings suggestive of significant illness. There was no clinical or laboratory evidence of renal, liver, or thyroid disease or abetalipoproteinemia.

Erythrocyte cations. Although the osmotic fragility of fresh In(Lu) erythrocytes was normal, incubation of these cells for 24 hours at 37 °C in plasma resulted in a significant resistance to osmotic lysis, in contrast to normal erythrocytes whose osmotic fragility increased after incubation in plasma. This striking difference in osmotic fragility is demonstrated in Fig 5, which compares the osmotic fragility curves of fresh and incubated red cells for an affected and a normal member of family S. Increased resistance to osmotic lysis was also observed when erythrocytes from the proposita of family C were incubated. Storage of In(Lu) erythrocytes for several hours at 4 °C did not affect these results.

Because resistance to osmotic lysis can occur as a consequence of cation depletion and cellular dehydration, the Na⁺ and K⁺ contents of fresh and incubated In(Lu) erythrocytes were determined (Table 3). Although In(Lu) and control cells had similar Na⁺ and K⁺ concentrations before incubation, significant K⁺ loss occurred during incubation of In(Lu) cells that was not seen in the controls. The K⁺ loss during incubation of erythrocytes from the family C proposita was partially offset by a gain in intracellular Na⁺. The total cation content of the two In(Lu) individuals was decreased, however, and may account for their relative resistance to osmotic lysis.

DISCUSSION

By use of a dilution of L21 mAb that strongly agglutinated normal erythrocytes and gave a weak or negative reaction

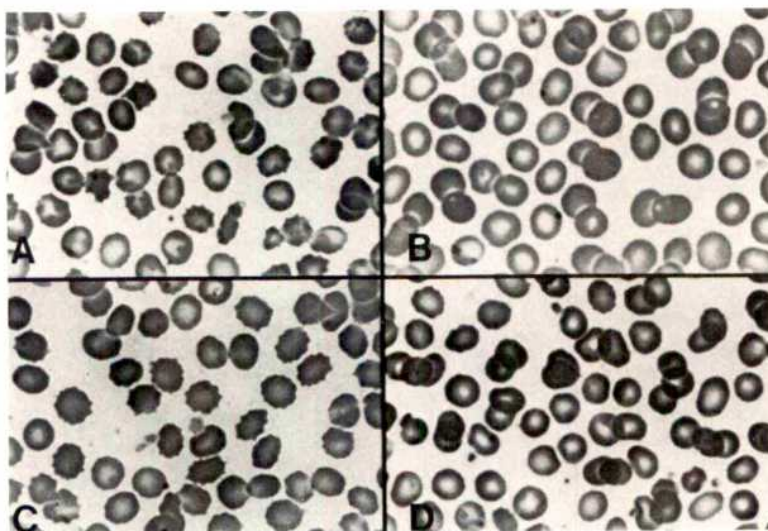


Fig 3. Photomicrographs (original magnification $\times 1000$) of Wright's-stained peripheral blood from family S. Acanthocytes are evident in In(Lu) individuals II-1 (A) and III-1 (C). Red cell morphology is normal in the non-In(Lu) members I-1 (B) and III-2 (D).

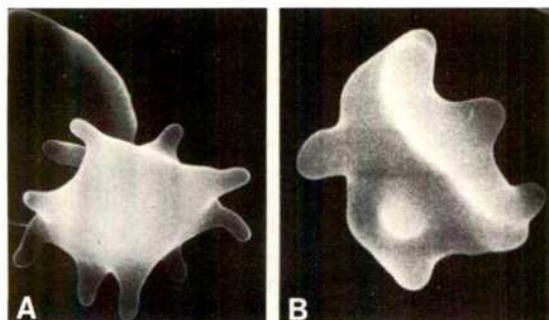


Fig 4. Scanning electron micrographs of washed erythrocytes from subject S, II-1 showing an acanthocyte in (A) (magnification $\times 10,350$) and a bizarre poikilocyte in (B) (magnification $\times 11,230$).

with In(Lu) cells, we were able to identify eight individuals with this phenotype among 42,000 donors tested in the Houston metropolitan region. Although the inheritance of this trait was studied in only two families, it is unlikely that any of these individuals represented the recessive Lu(a-b-) phenotype, because L21 reacts normally with erythrocytes of this phenotype. The frequency of the In(Lu) phenotype in our study, approximately 1:5,000, is similar to the frequency of 1:3,000 found by Shaw et al in Southeast England.¹⁸

The studies of these two families have revealed several new aspects of the In(Lu) phenotype: decreased agglutination of erythrocytes by Con A, and abnormal erythrocyte morphology and electrolyte metabolism. The reactivity of lectins with these erythrocytes was examined because two of the suppressed blood group antigens, P₁ and i, are known to have carbohydrate antigenic determinants.^{15,17} The diminished reactivity of In(Lu) cells with Con A is manifested primarily by a decreased strength of agglutination rather than a decreased agglutination titer. The diminished agglutination was not a consequence of abnormal erythrocyte morphology because the erythrocytes of the C family were not markedly abnormal in shape, and other samples of acanthocytic erythrocytes tested reacted normally with Con A and with the L21 and A3D8 mAbs.

In these two families the In(Lu) phenotype was associated with changes in erythrocyte morphology and osmotic fragility.

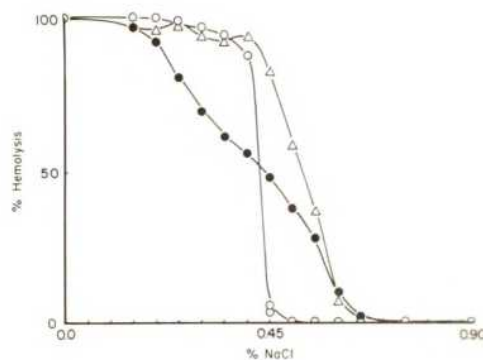


Fig 5. Osmotic fragility of In(Lu) erythrocytes from family S, subject II-1, compared to a normal family member, III-2. Nearly identical osmotic fragility is observed for fresh In(Lu) and control erythrocytes (O-O-O). In(Lu) RBC incubated at 37 °C for 24 hours in plasma (●-●-●) develop a population of cells resistant to osmotic lysis not seen in the incubated controls (Δ-Δ).

ty. In the S family acanthocytes and other bizarre poikilocytes were identified on routine peripheral blood films and confirmed by scanning electron microscopy of washed red blood cells. Another disorder of red blood cell antigen expression, the McLeod phenotype, is also associated with acanthocytosis.¹⁹ McLeod families, however, exhibit decreased expression of Kell red blood antigen, X-linked inheritance, and hemolysis. Other causes of acanthocytosis, including splenectomy, liver disease, and abetalipoproteinemia,²⁰ were also sought but were not identified. Thus the morphologic abnormalities seem to be another manifestation of the activity of the In(Lu) gene. The less striking erythrocyte changes observed in the proband of the C family (III-1), and the normal morphology of the two other affected members of this family suggests that the degree of red blood cell morphologic abnormality varies among different In(Lu) kindreds.

Although the two families described in this report differed in their degree of RBC morphologic change, they demonstrated similar changes in osmotic fragility. In both families the osmotic fragility of fresh In(Lu) erythrocytes was normal, whereas decreased osmotic fragility developed after incubation for 24 hours at 37 °C. The osmotically resistant incubated In(Lu) RBC were also K⁺-depleted when com-

Table 3. Cation Content* of Fresh and Incubated† In(Lu) RBC

Subjects	N‡	Na ⁺	K ⁺	Total (Na ⁺ + K ⁺)
Family S, II-1				
Fresh	3	12.2 ± 2.5	108.5 ± 12.9	120.6 ± 12.6
Incubated	2	15.4	79.0	94.4
Family C, III-1				
Fresh	3	16.6 ± 5.0	102.0 ± 11.6	122.2 ± 12.3
Incubated	3	27.7 ± 2.9§	63.3 ± 13.4§	91.1 ± 16.1§
Controls				
Fresh	6	12.6 ± 3.7	107.3 ± 13.0	119.9 ± 12.5
Incubated	5	19.1 ± 5.3	100.6 ± 15.7	119.8 ± 12.7

*Expressed as mmol/L packed RBC

†24 hours at 37 °C in plasma

‡Number of determinations

§Values differ from controls ($P \leq 0.05$)

pared to incubated normal controls. Because K^+ depletion usually results in dehydration of the erythrocyte, the K^+ loss observed after incubation of In(Lu) erythrocytes could explain the osmotic resistance, although RBC water content was not measured to verify this possibility.

What is the basic abnormality that underlies the In(Lu) phenotype? The studies of Telen et al¹⁰ have demonstrated that an 80-kD glycoprotein is immunoprecipitated by A3D8 from normal erythrocytes, and that only traces of this glycoprotein are immunoprecipitated from In(Lu) cells. It is not known whether the concentration of this protein is decreased in In(Lu) cells or whether the concentration of the protein is normal and epitope recognized by A3D8 is altered. It seems unlikely to us that this is the sole or principal abnormality of In(Lu) erythrocytes because of data pointing to abnormalities in other molecules. Band 3 is the major Con A-binding protein of human erythrocytes^{21,22} and it also carries the i antigenic determinant.²³⁻²⁵ The decreased expression of these receptors indicates an abnormality in the glycosylation of band 3. The P₁ antigenic determinant is expressed on glycolipids.¹⁵ In addition, there is evidence that the three monoclonal antibodies that react weakly with In(Lu) erythrocytes are directed at different epitopes. A3D8 and L21 agglutinate umbilical cord erythrocytes but H86 does not, and the receptor for H86 is protease-resistant but the receptors for L21 and A3D8 are protease-sensitive (refs 8 and 9, and Umeda and Marcus, unpublished observations).

We suggested previously¹⁵ that the basis of the In(Lu) phenotype is abnormal glycosylation of a common carbohydrate sequence that is present in many glycoproteins and in some glycolipids. The identification of decreased expression of the red cell Con A receptor lends further support to this

suggestion. Moreover, the occurrence of the determinants recognized by these monoclonal antibodies on several cell lineages, human erythrocytes, monocytes, and lymphocytes, and the presence of the L21 antigen on glycoproteins from several species, is more consistent with a carbohydrate than with a peptide determinant.

The designation In(Lu) reflects the first serological abnormality described in this phenotype, suppression of the Lu^a and Lu^b antigens. It is evident that the weak Lutheran antigens are only one of many biochemical and morphologic abnormalities displayed by this phenotype, and that the emphasis on the Lutheran antigens is misleading. Evidence for suppression of other high-frequency blood group antigens of In(Lu) erythrocytes was reported recently.²⁶ An important practical point is that the phenotype is more readily identified by the use of the new monoclonal antibodies than by screening for a weak Lu^b antigen. Human anti-Lu^b sera are very weak, as well as limited in supply, and it is difficult to determine accurately whether a weak Lu^b antigen represents the lower end of the normal distribution or a genetic abnormality. The availability of monoclonal antibodies for detection of the In(Lu) phenotype should facilitate biochemical studies of this interesting genetic abnormality.

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Divalent Cation-Dependent and Independent Surface Expression of Thrombospondin on Thrombin-Stimulated Human Platelets

By Martha L. Aiken, Mark H. Ginsberg, and Edward F. Plow

Thrombospondin (TSP), a platelet α -granule protein, becomes expressed on the surface of thrombin-stimulated platelets. The surface expression of this protein occurs through two distinct mechanisms. At low platelet concentrations (1×10^8 /mL), a divalent ion-independent, low-capacity mechanism predominates. At higher cell concentrations, a divalent ion-dependent, higher capacity mechanism prevails that can account for >90% of all the TSP surface expression measured. This mechanism

requires the presence of both calcium and magnesium (Ca + Mg). The dependence of the divalent ion-dependent surface expression on platelet concentration suggests that release of the molecule from the cell followed by its binding to the cell surface mediates this component of the endogenous TSP-platelet interaction. These data are consistent with a two-receptor model for the platelet surface expression of the endogenous TSP pool.

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THROMBOSPONDIN (TSP) is a trimeric glycoprotein comprised of 180-kD mol wt subunits.¹ It is a major protein of platelet α -granules²⁻⁵ and is synthesized by a variety of cells including monocytes,⁶ fibroblasts,⁷ endothelial cells,^{8,9} and megakaryocytes.¹⁰ Although the role of TSP in the function of these various cell types remains to be elucidated, several lines of evidence suggest that TSP is involved in platelet function. TSP binds to fibrinogen,¹¹⁻¹³ and fibronectin,^{13,14} which are involved in platelet adhesion and aggregation.¹⁵⁻¹⁸ TSP agglutinates fixed RBCs, platelets, or platelet membranes to one another.¹⁹⁻²¹ In addition, anti-TSP antibodies inhibit fibrinogen binding to thrombin-activated platelets.²² Finally, both monoclonal²³ and polyclonal antibodies²⁴ to TSP can inhibit the aggregation of human platelets.

The capacity of TSP to participate in platelet aggregation implies that the molecule must be available at the cell surface. Direct evidence for surface expression of TSP has been obtained by using cell surface-labeling approaches^{5,25-27} and from the binding of radiolabeled polyclonal and monoclonal antibodies to platelets.^{28,29} Both approaches suggest that thrombin stimulation of platelets results in surface expression of TSP as a consequence of its secretion from α -granules. Beyond these basic observations, there is limited information as to the regulation of TSP surface expression, and controversy exists. Using surface radioiodination approaches, Phillips and co-workers²⁶ indicated that TSP surface expression was calcium dependent, whereas George

and co-workers^{5,25} were able to radioiodinate TSP on the surface of thrombin-stimulated platelets in the presence of EDTA. In view of these uncertainties, we undertook a quantitative assessment of TSP surface expression using a monoclonal antibody to TSP. This antibody, TSP1-1, is a particularly interesting probe for such analyses as it has been shown to inhibit the hemagglutinating activity of TSP.³⁰ An additional impetus for these analyses has been our recent characterization of the interaction of exogenously added TSP and platelets.³¹ In the present study, we sought to determine if the two receptor systems that mediate the interaction of exogenously added TSP are also involved in regulating the surface expression of the molecule.

MATERIALS AND METHODS

Monoclonal antibody production and isolation. TSP was purified by a modification of the method of Lawler and co-workers.^{1,32} In brief, fresh human platelets (25 U) were stimulated with thrombin at 3 U/mL. A releasate fraction was obtained by centrifugation, and this fraction was applied to a heparin-Sepharose column. TSP was eluted, and this fraction and the nonbinding fraction were pooled and rechromatographed on heparin-Sepharose, using the same protocol. The second 0.45 mol/L of NaCl eluate containing TSP was pooled and frozen at -70°C . The typical yield of TSP was 1 mg from each unit of platelets.

TSP1-1 was produced using standard protocols. In brief, Balb/c mice were immunized with 25 μg of TSP followed by a second boost of 25 μg injected 2 weeks later. On the fourth and fifth day prior to fusion, the animals were given 40 μg of TSP intravenously (IV). Spleen cells from these mice were fused with the myeloma cell line P3X63Ag8.653 at a spleen-myeloma cell ratio of 5:1. TSP antibody-producing hybrids were detected with solid-phase radioimmunoassay as described,³³ coating microwells with 2.5 $\mu\text{g}/\text{mL}$ of TSP. The positive clones were subcloned by limiting dilution, retested in the solid-phase radioimmunoassay, and recloned.

The selected TSP1-1 clone was injected into pristane-primed mice, and ascites fluid was harvested during a 2-week period. The ascites fluid was characterized by electrophoresis on cellulose acetate strips and yielded a single major band in the γ region, consistent with the monoclonal nature of the antibody. By Ouchterlony analysis with class-specific monoclonal antibodies, TSP1-1 was determined to be an IgG2a antibody with a κ light chain.

TSP1-1 was purified essentially as described by Ey and colleagues.³⁴ In brief, ascites fluid (20 mL) containing TSP1-1 was incubated with Protein-A Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) for 18 to 20 hours at 4°C , with gentle agitation. Following the incubation, the slurry was centrifuged (120 g) for 5 minutes. The supernatant was removed, and the resin was resuspended in 0.1 mol/L of PO4, pH 8.0. The Protein A-

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Sephacryl was then washed with the equilibration buffer, and the bound protein was eluted in a stepwise fashion with 0.1 mol/L of sodium citrate at pH 5.5, 4.5, and 3.5. The pH 4.5 fraction, which contained TSP1-1, was dialyzed against phosphate-buffered saline (PBS), and the purified antibody was stored at -20°C . Typically, 1 to 2 mg of TSP1-1 was obtained from each milliliter of ascites. The antibody was $\geq 95\%$ homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

TSP1-1 was radiolabeled by a modified chloramine-T procedure³⁵ to a specific activity of $\sim 0.5 \mu\text{Ci}/\mu\text{g}$. Residual free iodine was removed by gel filtration on a PD-10 column (Pharmacia), equilibrated in and eluted with PBS. The protein concentration of the radiolabeled antibody was determined from the original protein concentration of the unlabeled protein, based on its absorption at 280 nm, and the precipitability of the radioactivity in 15% trichloroacetic acid prior to and after gel filtration. The precipitability of radioactivity in 15% trichloroacetic acid exceeded 98%, and protein recovery was routinely $>90\%$. The radiolabeled antibody (^{125}I -TSP1-1), as added to and as eluted from platelets (see below), exhibited a single band by SDS-PAGE (7.5% gels under nonreducing conditions) with a mobility corresponding to the isolated and nonlabeled antibody.

Platelet isolation. Washed human platelets were obtained as previously described.³⁶ In brief, 60 to 90 mL of fresh human blood were collected into acid-citrate-dextrose. This procedure was performed with the approval of our local Human Investigations Committee and the Department of Health and Human Services. The blood was centrifuged (120 g) for 20 minutes at 22°C . The platelet-rich plasma was removed and recentrifuged at 1,000 g at 22°C . The platelets were recovered as a pellet and resuspended in 2 mL of divalent ion-free Tyrode's buffer, pH 7.2, containing 2% bovine serum albumin, and subjected to gel filtration on Sepharose CL-2B ($2.5 \times 8 \text{ cm}$) (Pharmacia) equilibrated in the same buffer. The washed platelets were collected in $\sim 4 \text{ mL}$ and counted electronically in a Coulter Counter Model Zf (Coulter Electronics, Hialeah, FL). In some experiments, paraformaldehyde-fixed platelets were used. These were prepared as previously described.³⁷ Washed human platelets were incubated with thrombin at 0.25 U/mL for 5 minutes, and the agonist was inactivated with a tenfold excess of hirudin. Following platelet activation, paraformaldehyde was added to a final concentration of 0.05%. After 30 minutes at 22°C , 0.02 mol/L of NH_4Cl in 0.15 mol/L of NaCl, 0.02 mol/L of Tris, pH 7.2, was added in equal volume, and the platelets were centrifuged at 1,000 g for 15 minutes at 22°C . The supernatant was removed, and the platelets were resuspended in Tyrode's buffer, pH 7.2. After fixation, the cells were generally used within 24 hours but could be stored for 2 to 3 weeks at 4°C .

Platelet-binding assays. ^{125}I -TSP1-1 binding to platelets was measured as previously described³⁸ for the interaction of adhesive proteins with the cells. Typically, ^{125}I -TSP1-1 at 0.20 to 1.2 $\mu\text{mol}/\text{L}$ was incubated with washed human platelets at $1 \times 10^8/\text{mL}$ to $2 \times 10^9/\text{mL}$ in a final volume of 200 μL in Tyrode's-albumin buffer, pH 7.2. The platelets were then activated with thrombin at 0.25 U/mL. Following an incubation period at 22°C , triplicate 50- μL aliquots of the incubation mixture were layered onto 20% sucrose and centrifuged in a Beckman microfuge for 2.5 minutes. The tips of the tubes containing the platelets were amputated and counted. By means of the specific activity of the radiolabeled ligand, the number of antibody molecules bound per platelet was determined. Nonspecific binding, N_1 , as determined using the "Ligand" program,³⁹ ranged from 0 to $3 \times 10^{-2}\%$ of the TSP1-1 input concentration and was verified experimentally at low-input concentrations of ^{125}I -TSP1-1 in the presence of a large excess of nonlabeled antibody. All data presented represent specific binding of the antibody.

Immunoblotting analyses. Plasmin (KabiVitrum, Stockholm)

was added to human TSP to a final concentration of 0.5 U/mL with TSP at 1 mg/mL. The data shown in this study was obtained with a 3-hour digest at 37°C . The sample was added to an equal volume of Laemmli sample buffer³⁹ containing 2% 2-mercaptoethanol and was boiled. These samples were then subjected to PAGE in the buffer system of Laemmli,⁴⁰ using a 10% resolving gel with a 3.3% stacking gel. After electrophoresis, the protein was transferred from the gel to nitrocellulose for 1 hour at a constant current of 275 mA.⁴¹ Nonspecific binding to the nitrocellulose was reduced by incubation with a phosphate buffer containing casein⁴² for 1 hour at 22°C . TSP1-1 ascites fluid, at a dilution of 1:250 in the casein-blocking buffer, was added to the nitrocellulose and incubated for 2 hours. The nitrocellulose sheets were then washed three times, each time for 10 minutes with the same buffer. Immunochemically purified ^{125}I -radiolabeled anti-mouse IgG diluted in the phosphate-casein buffer (0.5 $\mu\text{g}/\text{mL}$) was added to the washed nitrocellulose sheets. Following a 2-hour incubation at 22°C , three 10-minute washes with the blocking buffer were performed; last, the sheets were washed with PBS. The bound radiolabeled anti-mouse IgG was detected by autoradiography of the dried sheets; mol wts were estimated from the mobilities of mol wt standard proteins applied to the gel.

RESULTS

Antibody specificity. TSP1-1 was specific for TSP based on the following criteria. The antibody reacted with TSP in a solid-phase radioimmunoassay, and this interaction could be inhibited by purified TSP in the fluid phase. In similar analyses, the antibody did not react with fibrinogen or fibronectin. In immunoblotting analyses, the antibody reacted with a single component in platelet lysates with the same mobility as purified TSP, and in immunofluorescence the antibody stained in a pattern consistent with α -granule localization.⁴³

To characterize further the recognition specificity of TSP1-1, purified TSP was subjected to plasmin digestion in the presence of Ca. The resulting samples were subjected to electrophoresis on 10% polyacrylamide gels under reducing conditions and then electrophoretically transferred onto nitrocellulose. The gel of nondigested TSP and the digest is shown in the left panel of Fig 1. Nondigested TSP (lane 1) exhibited a single, major band with an estimated mol wt of 180 kd, consistent with the properties of the TSP subunits in the Laemmli gel system.⁴⁴ The digest (lane 2) contained two major bands at estimated mol wts of 150 kd and 30 kd. Based on the studies of Lawler and Slater⁴⁵ the 30-kd fragment corresponds to the heparin binding fragment from the amino-terminal region of TSP. When the transfers were subjected to immunoblotting analyses with TSP1-1, the antibody reacted with the intact subunits of TSP (right panel, lane 3). In the plasmin digest (lane 4), TSP1-1 reacted with the 30-kd but not the 150-kd fragment of TSP. This suggests that the TSP1-1 epitope resides in the heparin binding domain of TSP.

Binding of TSP1-1 to platelets. TSP1-1 was radioiodinated and used to assess the availability of the recognized epitope on the surface of thrombin-stimulated and nonstimulated platelets. In the initial set of experiments, ^{125}I -TSP1-1 was used at an input concentration of 1 $\mu\text{mol}/\text{L}$ (determined by subsequent experiments to be a saturating dose of the antibody) with the cells at $2 \times 10^8/\text{mL}$. The data shown in

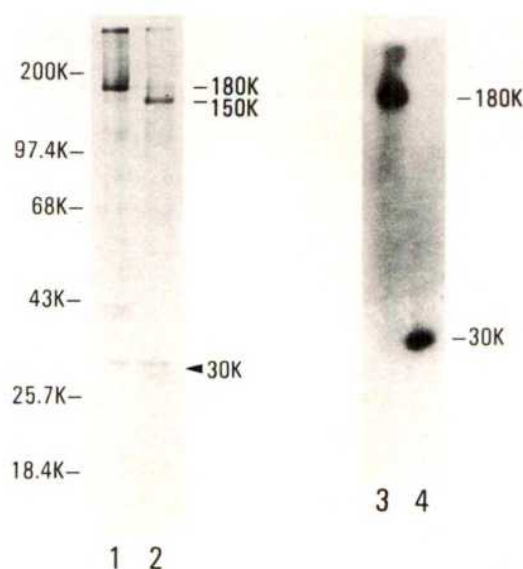


Fig 1. TSP was digested with 0.5 U plasmin per mg of TSP for 3 hours at 37 °C in the presence of 2 mmol/L of Ca. Both the intact protein and the digest were subjected to immunoblot analysis using TSP1-1 to determine which TSP domain contains the TSP1-1 epitope. Left panel, 10% Laemmli gel stained with Coomassie Blue: lane 1, intact TSP; lane 2, plasmin digest of TSP. Right panel, autoradiogram of the immunoblot with TSP1-1: lane 3, intact TSP; lane 4, plasmin digest of TSP. Arrow indicates peptide generated by plasmin cleavage. The contaminant in the starting sample in this mol wt range did not appear to be affected by the digestion with plasmin.

Table 1 were obtained after a 60-minute incubation and establish two major points regarding surface expression of TSP. First, surface expression of TSP is increased when the platelets are stimulated. In the presence of Ca + Mg, thrombin-stimulated platelets bound 15 times more TSP1-1 than did nonstimulated platelets in the presence of PGE1 and theophylline. In addition, when blood was drawn directly into PGE1 and theophylline and the inhibitors were maintained throughout the platelet isolation, TSP1-1 binding was further reduced. When ^{125} I-TSP1-1, at 0.3 μ mol/L, was incubated for 30 minutes with platelets prepared from blood drawn into PGE1 and theophylline, <200 antibody molecules were bound per platelet in the presence of EDTA or Ca + Mg as compared with 1,000 molecules bound per platelet when the inhibitors were added directly to the isolated cells. Second, thrombin-stimulated surface expression of TSP was detected in the presence of EDTA. The

Table 1. Surface Expression of TSP on Resting and Thrombin-Stimulated Platelets

Divalent Ion Condition	^{125} I-TSP1-1 Bound (Molecules/Platelet)	
	Resting	Stimulated
EDTA	850 $\pm$ 20	3,100 $\pm$ 500
Ca + Mg (2 mmol/L of each)	1,100 $\pm$ 340	16,000 $\pm$ 4,200

^{125} I-TSP1-1 was added at a saturating dose, 1 μ mol/L, to resting or thrombin-stimulated (0.25 U/mL) fresh platelets at 2×10^8 /mL, and binding was measured after 60 minutes. PGE1 and theophylline were present in the resting platelet preparation at 1 μ g/mL and 1 mmol/L, respectively.

extent of surface expression was reduced relative to that observed in the presence of Ca + Mg, but still constituted a significant increase, approximately fourfold, as compared with nonstimulated cells.

With these data suggesting that the divalent ions may regulate the extent of TSP surface expression, the divalent ion conditions for surface expression were further investigated. With ^{125} I-TSP1-1 added at its K_d (0.2 μ mol/L), thrombin-stimulated surface expression of TSP was measured in the presence of Ca + Mg, Ca or Mg, Mg-EGTA, or EDTA (Fig 2). In the absence of divalent ions (EDTA), or in the presence of Mg-EGTA or Ca, the binding of TSP1-1 was measurable but remained at a relatively low level with 3,000 to 3,500 molecules of antibody bound per platelet. Addition of Mg or Ca alone at concentrations as high as 3.8 or 4 mmol/L,⁴⁶ respectively, failed to augment TSP surface expression. When 2 mmol/L of Ca + 2 mmol/L of Mg were present, a fourfold increase (3,500 v 17,400 molecules of TSP1-1 per platelet) in TSP surface expression occurred. A similar increase in antibody binding was also observed with the addition of Mg without chelation of endogenous Ca, suggesting that the platelets may provide a sufficient quantity of this ion for maximal TSP surface expression.

TSP1-1 binding to thrombin-stimulated platelets reached steady state at similar times in the presence of Ca + Mg and EDTA. As shown in Fig 3, under both conditions, apparent equilibrium binding was observed in 60 minutes. At a single concentration of TSP1-1 (0.25 μ mol/L) as in Fig 3, increased binding could reflect an increase in the affinity of the antibody for TSP in the presence of Ca + Mg rather than reflect an increase in the quantity of TSP on the platelet surface. Therefore, the binding of TSP1-1 added at varying concentrations to thrombin-stimulated platelets was assessed

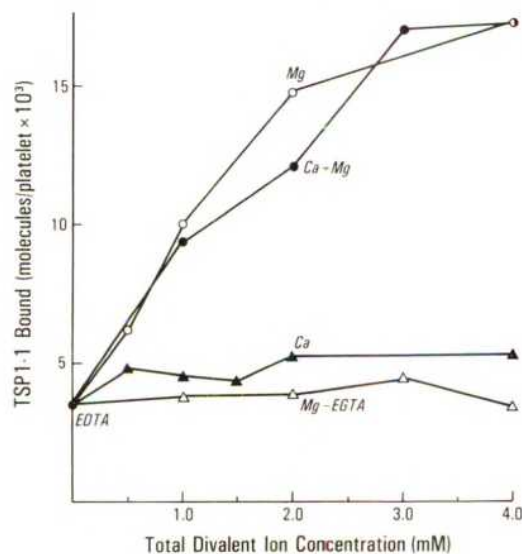


Fig 2. ^{125} I-TSP1-1 was incubated with thrombin-stimulated (0.25 U/mL) platelets at a platelet concentration of 2×10^8 /mL. The final concentration of EDTA was 5 mmol/L. The concentration of Mg + Ca ranged from 0.5 to 4 mmol/L and the concentration of Mg-EGTA ranged from 5 mmol/L of EGTA–1 mmol/L of Mg to 5 mmol/L of EGTA–4 mmol/L of Mg, at which point the calculated free Mg concentration was ~3.8 mmol/L.⁴⁷

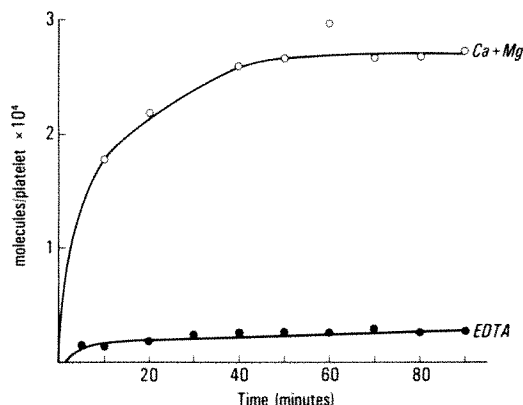


Fig 3. Platelets were stimulated with thrombin (0.25 U/mL) for 10 minutes prior to fixation with paraformaldehyde. Following a wash of the platelets with Tyrode's buffer, ^{125}I -TSP1-1 (0.25 $\mu\text{mol/L}$) was added to the cells ($2 \times 10^8/\text{mL}$) for varying periods of incubation (5 to 90 minutes). Ca + Mg (2 mmol/L of each) present during stimulation (○), EDTA (5 mmol/L) present during stimulation (●).

under various divalent ion conditions. In these experiments, both fresh and paraformaldehyde-fixed washed platelets were used. The affinity of TSP1-1 for platelet-bound TSP may be different than that for TSP in solution, and the free TSP may compete with a high affinity for TSP1-1 binding, resulting in an underestimation of the affinity of TSP1-1 for platelet-bound TSP. Therefore, platelets were stimulated, fixed with paraformaldehyde, and then washed to remove free TSP prior to the assay. Binding isotherms were constructed by adding increasing concentrations of ^{125}I -TSP1-1 (0.2 to 1.2 $\mu\text{mol/L}$) to thrombin-stimulated fixed or nonfixed platelets at $2 \times 10^8/\text{mL}$. The results shown in Fig 4 were obtained in the presence of EDTA, and saturation of antibody binding is apparent for both the fresh and fixed platelets. Such data were subjected to Scatchard analyses, and the binding parameters derived are summarized in Table 2. The affinity of TSP1-1 for platelet-bound TSP showed little variation with divalent ion conditions; however, the number of TSP1-1 binding sites was greater than eightfold higher in the presence of Ca + Mg as compared with EDTA. The slight shift in affinity of the antibody for fixed platelets as compared with fresh platelets suggests that the antibody does not have a significantly higher affinity for TSP in solution than for platelet-bound TSP.

The ability of EDTA to reverse the Ca + Mg-dependent surface expression of TSP on thrombin-stimulated platelets was also investigated (Table 3). Cells were activated with thrombin for 10 minutes in the presence of Ca + Mg, a condition that induced a full secretory response as measured by β -thromboglobulin release. EDTA was then added to a final concentration of 10 mmol/L. With an input concentration of ^{125}I -TSP1-1 of 1.0 $\mu\text{mol/L}$, EDTA reduced binding of the antibody to platelets by ~65% as compared with that observed in the presence of Ca + Mg, and this reduction approached the level of surface expression noted on cells activated in the presence of EDTA. These results indicate that EDTA can reverse the increased TSP surface expression that occurs in the presence of Ca + Mg. This reversal was

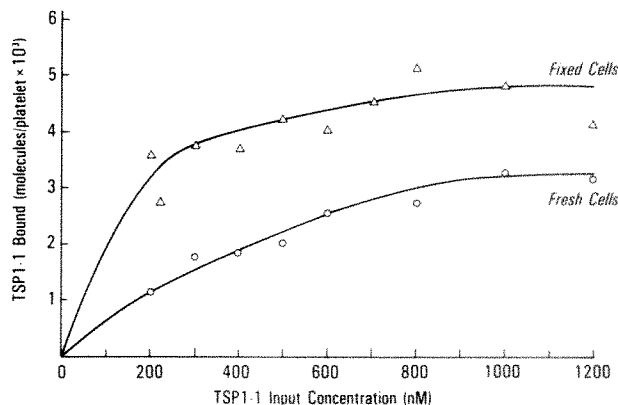


Fig 4. Increasing concentrations of ^{125}I -TSP1-1 (0.2 to 1.2 $\mu\text{mol/L}$) were incubated with either fresh or paraformaldehyde-fixed platelets. The fresh cells were stimulated with thrombin (0.25 U/mL) at a cell concentration of $4 \times 10^8/\text{mL}$ prior to the assay. To prepare the fixed cells, platelets were stimulated with thrombin (0.25 U/mL) at a cell concentration of $1.5 \times 10^9/\text{mL}$ and fixed with 0.05% paraformaldehyde and then washed with Tyrode's buffer to remove free TSP. Both sets of cells were diluted to a final concentration of $2 \times 10^8/\text{mL}$ in the binding assay.

also observed with γ -thrombin as the stimulus of platelet secretion (Table 3).

TSP may become surface expressed through its secretion into the extracellular milieu and its subsequent binding to the cell surface. Accordingly, platelet concentration at the time of thrombin stimulation would determine the concentration of TSP available for binding the cells; therefore, the extent of TSP surface expression would be dependent on platelet concentration. This prediction has been examined experimentally. Platelets, at concentrations from $1 \times 10^8/\text{mL}$ to $2 \times 10^9/\text{mL}$, were stimulated with thrombin in the presence of either Ca + Mg or EDTA and then fixed with paraformaldehyde. The cell concentration was then adjusted to $2 \times 10^8/\text{mL}$, and surface expression of TSP was measured using a saturating dose of TSP1-1 (1.1 $\mu\text{mol/L}$) as a probe. The results are summarized in Table 4. The TSP surface expression increased sixfold and was 90% divalent ion dependent at the highest cell concentration tested ($2 \times 10^9/\text{mL}$). Thus, as the platelet concentration at the time of thrombin stimulation increased, resulting in higher concentrations of secreted TSP, TSP surface expression also increased in the presence of Ca + Mg. This effect was not noted in the presence of EDTA. Surface expression varied by only 14% at the lowest and highest platelet concentrations used in the presence of EDTA as compared with a >700% difference in the presence of Ca + Mg.

DISCUSSION

TSP1-1 recognizes an epitope in the heparin-binding domain of TSP. Because this antibody has been previously shown to inhibit the lectinlike activity of TSP,³⁰ this localization suggests that the amino-terminal region of the molecule is involved in this function, consistent with the data of other researchers,⁴⁷ and that this region is available on the platelet surface in the presence or absence of divalent ions. Using TSP1-1 to measure surface expression of the molecule, we

Table 2. Summary of TSP1-1 Binding Parameters

Parameter	Platelets			
	Fresh		Fixed	
	Ca + Mg	EDTA	Ca + Mg	EDTA
Dissociation constant K_d (nmol/L)	165 $\pm$ 55	540 $\pm$ 220	200 $\pm$ 70	90 $\pm$ 80
Binding sites (TSP1-1 molecules/platelet)	17,000 $\pm$ 2,000	5,200 $\pm$ 1,300	42,000 $\pm$ 9,000	5,000 $\pm$ 1,500

Varying concentrations of ^{125}I -TSP1-1 were added to platelets at $2 \times 10^8/\text{mL}$ in the presence of Ca + Mg (2 mmol/L of each) or EDTA (5 mmol/L). The fresh platelets were stimulated with thrombin (0.25 U/mL) at a concentration of $4 \times 10^8/\text{mL}$ and diluted to $2 \times 10^8/\text{mL}$ in the assay, whereas the paraformaldehyde-fixed platelets were stimulated with thrombin (0.25 U/mL) prior to fixation at a concentration of $1.5 \times 10^8/\text{mL}$ and diluted to $2 \times 10^8/\text{mL}$ in the assay.

found that maximal exposure of TSP on thrombin-stimulated platelets requires the presence of both Ca + Mg. In the presence of Ca alone, EDTA or Mg-EGTA surface expression was reduced relative to the Ca + Mg condition. Mg alone, but not Mg-EGTA, was as effective as Ca + Mg in supporting TSP surface expression, suggesting that the endogenous pool of Ca supplied by the platelets is a sufficient source of this ion for optimal TSP exposure.

The enhanced TSP1-1 binding in the presence of Ca + Mg could be an effect of the combination of divalent ions on the affinity of TSP1-1 for the molecule, or on platelet secretion, which would control the availability of TSP, or directly on the quantitative expression of TSP on the platelet surface. The affinity of TSP1-1 for the molecule on the platelet surface in the presence of Ca + Mg or EDTA was quite similar. Using purified TSP in a fluid-phase radioimmunoassay or in a solid-phase radioimmunoassay with TSP coupled to plastic microtiter wells, we found that TSP1-1 binding also was similar under varying divalent ion conditions (unpublished observations, August 1985). Moreover, the affinity of TSP1-1 for platelets in the presence or absence of Ca + Mg did not vary significantly when measured with either fresh or fixed platelets. Therefore, differences in the affinity of TSP1-1 for TSP in the presence of Ca + Mg as contrasted to other divalent ion conditions does not appear to be the basis for its increased binding. That Ca + Mg enhances platelet secretion of TSP also appears to be excluded. When platelets were stimulated in the presence of Ca + Mg and then EDTA was added, TSP1-1 binding was reduced and approached the levels that occurred when cells were stimulated in the presence of EDTA. Thus, it

seems likely that Ca + Mg directly increases the number of TSP molecules, or more precisely the number of TSP1-1 epitopes, on the surface of thrombin-stimulated platelets. It is well established that TSP is a Ca-binding protein and that the removal of Ca results in a conformational change of the molecule.⁴⁴ Whether Mg has an additional effect on the molecule, the platelet, or both remains to be demonstrated. It is noteworthy in this regard that Dixit and colleagues¹² reported that the association of TSP and fibrinogen is also optimal in the presence of both divalent ions, and a preliminary report⁴⁸ also suggests that TSP binding to macrophages is optimal in the presence of both divalent ions.

Removal of all divalent ions with EDTA did not entirely abolish TSP surface expression. This result suggests that existence of at least two distinct mechanisms for surface expression of TSP. One pathway is divalent ion independent, and the second is dependent on Ca + Mg. The relative contribution of these two pathways to TSP surface expression is dependent on platelet concentration. When platelets were stimulated at a low cell concentration ($1 \times 10^8/\text{mL}$), ~60% of the TSP surface expression was divalent ion independent. As the cell concentration was increased, the quantity of TSP surface expressed by the divalent ion-independent mechanism remained relatively constant, whereas the Ca + Mg-dependent surface expression increased until it constituted ~90% of the TSP surface expression at the highest platelet concentration tested ($2 \times 10^9/\text{mL}$). The

Table 3. Effect of EDTA on TSP Surface Expression

Ion Condition	(TSP1-1 Molecules/Platelet)	
	γ -Thrombin (10 U/mL)	α -Thrombin (0.25 U/mL)
Ca + Mg (2 mmol/L of each)	17,000	19,000
EDTA* (10 mmol/L)	3,100	4,300
EDTA† (10 mmol/L)	6,300	6,100

^{125}I -TSP1-1 (1.0 $\mu\text{mol/L}$) was added to stimulated, fresh platelets at $2 \times 10^8/\text{mL}$ in the presence of Ca + Mg or EDTA. In one set of samples, the platelets were stimulated in the presence of Ca + Mg with the addition of EDTA after a 10-minute incubation.

*EDTA present at time of stimulation.

†EDTA added after the cells were incubated with the stimulus for 10 minutes in the presence of Ca + Mg.

Table 4. Effect of Cell Concentration on TSP Surface Expression

Platelets* (Cells/mL)	TSP1-1 Bound (Molecules/Platelet)		
	Ca + Mg (2 mmol/L of each)	Total† (%)	EDTA (5 mmol/L)
1×10^8	7,100	40	4,300
5×10^8	22,000	86	3,200
1×10^9	36,300	85	5,600
2×10^9	60,000	92	5,000

Platelets at varying concentrations were stimulated with thrombin (0.25 U/mL) for 10 minutes prior to fixation with paraformaldehyde. The fixed cells were then centrifuged and resuspended in Tyrode's buffer at a final cell concentration of $2 \times 10^8/\text{mL}$ in the assay. The cells were incubated with ^{125}I -TSP1-1 (1.1 $\mu\text{mol/L}$) for 60 minutes.

*Platelet concentration at the time of stimulation.

†If it is assumed that TSP1-1 binding in the presence of Ca + Mg represents the sum of divalent ion-dependent and -independent binding or 100% of the total TSP surface expression, this is the percentage attributable to a divalent ion-dependent mechanism.

existence of these two independent pathways may reconcile previous data^{26,27} regarding the divalent ion dependence of TSP surface expression. From our data, it is clear that both mechanisms can occur and that platelet concentration can be an important variable in determining the relative contribution of each mechanism. The basis for the effect of platelet concentration remains to be established. The most direct explanation is that platelet concentration at the time of thrombin stimulation determines the concentration of available TSP in solution. At 10^8 or 10^9 platelets/mL the theoretical concentration of secreted TSP would be 4 or 40 nmol/L, respectively.⁴⁹ If the divalent ion-independent mechanism is of high affinity and low capacity such that these sites are saturated at 4 nmol/L of TSP, an increase in the TSP concentration would not increase the number of molecules bound per platelet. In contrast, if the Ca + Mg-dependent reaction is of lower affinity and higher capacity, a tenfold increase in available TSP concentration could increase surface expression significantly. As noted below, this interpretation is consistent with our previous data³¹ on the binding of exogenously added TSP to platelets. More complex explanations, however, cannot be excluded. For example, the concentration of other secreted platelet constituents could also influence TSP binding to platelets, or the state of TSP polymerization⁵⁰ could be influenced by Ca + Mg.

We recently showed that the binding of exogenously added TSP to platelets also involves two distinct mechanisms.³¹ One of these mechanisms is divalent ion dependent. It occurs with relatively low affinity ($K_d = 250$ nmol/L) and high capacity ($35,600 \pm 9,600$ sites per cell). The divalent ion-independent mechanism for binding of exogenously added TSP is of higher affinity ($K_d = 50$ nmol/L) and lower capacity ($3,100 \pm 1,000$ sites per platelet). Thus, the predictions regarding the relative affinity and capacity of the divalent ion-independent and the Ca + Mg-dependent mechanism of

surface expression are consistent with the binding of exogenously added TSP. The data from the two studies are not entirely in agreement. First, the divalent ion-dependent binding of exogenous TSP could be demonstrated in the presence of Ca or Mg alone. Second, since the divalent ion-independent surface expression of TSP changed minimally with platelet concentration, a very high-affinity interaction, higher than a K_d of 50 nmol/L, is predicted. If endogenous and exogenous TSP bind to the same receptors, when exogenous TSP is added it must compete with secreted endogenous TSP for binding sites, which would result in a lower apparent affinity for TSP binding. Thus, the difference in affinity of exogenous TSP for the divalent ion-independent binding site ($K_d = 50$ nmol/L) and the affinity of endogenous TSP for the platelet surface in the presence of EDTA (apparent $K_d < 4$ nmol/L based on estimated available TSP concentrations) may be artifactual. It is also possible that the differences found between the binding of exogenous TSP and the surface expression of endogenous TSP is due to subtle changes arising during purification of the molecule.

When blood was collected into PGE1 and theophylline and these inhibitors were maintained throughout the platelet isolation, <200 molecules of TSP1-1 bound per platelet. Based on the affinity and the plasma concentration of TSP, it is predicted that <250 molecules of TSP should be present on the surface of nonstimulated platelets. The practical implications of these results is that detection of TSP on the platelet surface with monoclonal antibodies such as TSP1-1 could prove to be a useful marker for platelet activation. Extensive and detailed investigations will be necessary to assess this possibility.

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Preclinical Assessment of Purging With VP-16-213: Key Role for Long-Term Marrow Cultures

By Brian H. Kushner, Jong-Hyo Kwon, Subhash C. Gulati, and Hugo Castro-Malaspina

An evaluation of the effects of VP-16 on normal human marrow cells and representative lymphoma-leukemia cell lines was performed to assess this agent's applicability to ex vivo marrow purging. Tumoricidal dose curves were defined using malignant lymphoid (SK-DHL2 and Reh) and myeloid (HL-60) cells admixed with a 20-fold excess of irradiated marrow cells to simulate a borderline remission marrow. One-hour treatments yielded ID_{50} of $<5 \mu\text{mol/L}$ of VP-16 for clonogenic units from each cell line; rare-to-zero clonogenic units survived exposure to 50 to 100 $\mu\text{mol/L}$. CFU-Mix, BFU-E, and CFU-GM were equal in their sensitivity to VP-16 (ID_{50} s 25 to 30 $\mu\text{mol/L}$). Marrows treated with 75 $\mu\text{mol/L}$ were completely depleted of these colony-forming cells but produced CFU-GM in one-stage long-term

marrow cultures (LTMCs). This dose had little adverse effect on the proliferative capacity of marrow stromal progenitors, as measured by CFU-F (ID_{50} 271 $\mu\text{mol/L}$) and by the unperturbed development of adherent layers in LTMCs. Furthermore, these stromal layers were able to support hematopoiesis as well as controls in co-culture experiments with autologous marrow cells (two-stage LTMCs). In conclusion, doses of VP-16 that cleanse marrow of lymphoma-leukemia cells spare hematopoietic and stromal progenitors as demonstrated by LTMCs. These data favor the use of VP-16 in the clinical autotransplant setting.

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THE FEASIBILITY of eliminating neoplastic cells from harvested bone marrow¹ has spurred the use of myeloablative chemoradiotherapy with autologous marrow rescue. Immunologic²⁻⁴ or pharmacologic^{1,5,6} manipulations exploit differential features or sensitivities of malignant cells to maximize antineoplastic impact while preserving hematopoietic stem cells needed for hematologic recovery post-transplant. Noxious effects on marrow stromal cells in the autograft pose an additional concern.⁷ The intravenous transplantability of these cells and the replacement of host stroma by cells of donor origin remain unsettled points⁸⁻¹⁰ but may prove to be key issues if evolving myeloablative regimens are found to impair the marrow microenvironment's hematopoietic supportive functions.^{11,12}

Purging methods differ in terms of these objectives. For example, although monoclonal reagents and complement spare normal hematopoietic and stromal cells, their antineoplastic effect may be blunted by excess marrow.^{2,7,13} Conversely, chemopurification reliably eliminates tumor cells despite excess marrow, but it also depletes hematopoietic^{6,14,15} and, to a lesser degree, marrow stromal progenitors.¹⁴

VP-16-213 (VP-16), an active anti-lymphoma/leukemia derivative of podophyllotoxin, arrests cells in late S or early G₂ phase.^{16,17} A linear relation between VP-16 dosage and greater antineoplastic effect,¹⁸ plus its in vitro activity, make VP-16 an attractive candidate for marrow purging. To explore this potential, we correlated the effects of VP-16 on the hematopoietic and stromal compartments of human bone marrow with its toxicity on representative lymphoma-leukemia cell lines. The studies support a role for VP-16 in purging and highlight the usefulness of long-term marrow cultures (LTMCs) for preclinical assessments of purging regimens.

MATERIALS AND METHODS

Cells and Incubation Procedure

Heparinized bone marrow aspirates from the iliac crests of healthy volunteers who gave informed written consent were centrifuged at 200 g for 10 minutes. Buffy coat cells were then suspended in α -medium (Flow Laboratories, Hamden, CT) with 10% heat-inactivated fetal calf serum (FCS).

Neoplastic targets included: (a) SK-DHL2, a B cell lymphoma line with an 8;14 translocation¹⁹; (b) a common ALL antigen

(CALLA+), non-T, non-B acute lymphoblastic leukemia cell line²⁰; and (c) HL-60, a promyelocytic leukemia cell line.²¹ Cells were kept at 37 °C in a humidified 5% CO₂ atmosphere and were maintained in exponential growth in RPMI 1640 (GIBCO, Grand Island, NY) with 10% FCS, 1% penicillin:streptomycin:neomycin (PSN), and 1% glutamine (GIBCO).

VP-16 (Bristol, Syracuse, NY) was diluted with calcium- and magnesium-free phosphate-buffered saline (PBS) immediately before use. Marrow buffy coat cells at 5 or 20 $\times 10^6/\text{mL}$ of α -medium with 10% FCS, and neoplastic cells (alone or admixed with a 20-fold excess of irradiated [4,000 rad] marrow cells) at 1 $\times 10^6/\text{mL}$ of RPMI 1640 with 10% FCS, were incubated at 37 °C for 1 hour with graded doses of VP-16 or medium control, washed three times and collected for use.

Culture Techniques

Lymphoma-leukemia colony-forming cells. Because of preliminary studies that showed suboptimal colony size and reduced plating efficiency when treated neoplastic cells were cultured at more than 10⁵/mL, 200 and 10³ untreated or 10³ and 10⁴ treated lymphoma-leukemia cells in 1 mL of culture medium were plated in quadruplicate onto 1-mL underlayers prepared in 35-mm dishes (Lux Scientific Co, Newburg, CA). The culture medium consisted of 1.08% methylcellulose in RPMI 1640 with 20% FCS, 1% PSN, and 1% glutamine; the underlayers consisted of 0.5% agar in McCoy's 5A medium with 20% FCS and 1% PSN, but no feeder cells. Cultures were incubated at 37 °C in a humidified 5% CO₂.

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atmosphere and scored for colonies (>50 cells/aggregate) on day 10.

Granulocyte-macrophage colony-forming cells. Control or VP-16-treated marrow buffy coat cells were cultured as described¹⁴ in quadruplicate in 35-mm dishes (Corning Glass Works, Corning, NY) at 2×10^5 /mL of 0.3% agar in supplemented McCoy's 5A medium and 10% FCS; in each dish, 0.1 mL of medium conditioned by the 5637 bladder carcinoma cell line stimulated colony formation.²² CFU-GM (>50 cells/aggregate) were scored on day 7.

Multilineage and erythroid colony-forming cells. Mononuclear cells were recovered from control and VP-16-treated marrow buffy coat cells by neutral density centrifugation in an isotonic sterile Percoll solution (1.074 g/mL, 270 mOsm) (Pharmacia, Piscataway, NJ) and were cultured, as described,²³ in quadruplicate in 35-mm dishes (Lux) at 0.5 to 1.0×10^5 /mL of 1% methylcellulose in Iscove's modified Dulbecco's medium (IMDM) with 30% human plasma, 10% phytohemagglutinin-stimulated leukocyte-conditioned medium, 5×10^{-5} mol/L of 2-mercaptoethanol, and 1 U of human recombinant erythropoietin (AMGen, Thousand Oaks, CA). CFU-Mix and BFU-E were scored on day 14. Random colonies were plucked and stained with Wright-Giemsa solution for verification of cellular composition.

CFU-GM generation in one-stage LTMCs. Twenty $\times 10^6$ control or VP-16-treated marrow buffy coat cells suspended in 10 mL of medium were inoculated into T-25 flasks (Corning) which were gassed with 5% CO₂ in air, and kept at 33 °C. The LTMC medium consisted of supplemented McCoy's 5A medium,¹⁴ 12.5% horse serum, 12.5% FCS, and 10^{-6} mol/L of hydrocortisone sodium succinate. On day 5, supernatants were purged of erythrocytes and mature myeloid cells by Percoll separation; the mononuclear cells were returned to their flasks in fresh medium. Half of the supernatant was removed weekly and the cells in suspension were assayed for CFU-GM. At week 7, adherent layers were suspended, as described,¹⁴ using a nontoxic 0.01% (wt/vol) Ca²⁺-free and Mg²⁺-free trypsin (GIBCO) solution to determine their CFU-Mix, BFU-E, and CFU-GM content.

Fibroblast colony-forming cells. Five $\times 10^6$ control or VP-16-treated marrow buffy coat cells in 20-mL of α -medium with 20% FCS were set up in T-75 flasks (Corning), which were gassed with 5% CO₂ in air and kept at 37 °C. Medium was renewed on day 4. Cultures were stopped on day 10, stained with Wright-Giemsa solution, and scored for CFU-F (>50 cells/aggregate).²⁴

Stromal layers in LTMCs. To monitor the kinetics of stromal layer development, the extent of flask coverage by an interlocking network of cells was estimated weekly by phase microscopy. Cellular composition was assessed *in situ* by immunofluorescence methods as described.¹⁴ Fibroblasts were identified by rabbit antibodies to fibronectin (Bethesda Research Laboratory, Gaithersburg, MD) and to type III collagen (kindly provided by Dr H. Furthmayr, Yale University). Endothelial cells and macrophages were identified by mouse monoclonal antibodies (Australian Monoclonal Development, Antammon, Australia) to factor VIII-related protein and to human monocytes/macrophages, respectively. Lipid-containing cells were distinguished by their morphology.

To assess the hematopoietic supportive capacity of stromal layers established from VP-16-treated marrow, all suspension cells were removed from 5-week-old LTMCs and 15×10^6 freshly isolated nonadherent light-density (NAL) autologous marrow cells were added. NAL cells were obtained by Percoll separation followed by 12-hour adherence in T-75 flasks. The two-stage LTMCs were demopulated weekly for determination of CFU-GM content. Controls included: (a) cell-free LTMC medium on stromal layers from untreated and VP-16-treated marrow, (b) NAL cells in LTMC medium without stromal layers, and (c) NAL cells on stromal layers from untreated marrow.

RESULTS

Effect of VP-16 on Clonogenic Lymphoma-Leukemia Cells

VP-16 exerted a dose-dependent killing of clonogenic lymphoma-leukemia cells (Fig 1). In the absence or presence of a 20-fold excess of irradiated marrow cells during the 1-hour incubation period, the dose of VP-16 that inhibited 50% (ID₅₀) and 90% (ID₉₀) of colony growth for all three cell lines was <5 μ mol/L and <10 μ mol/L, respectively. Rare-to-zero clonogenic SK-DHL2 and Reh cells survived treatments with 50 to 100 μ mol/L of VP-16; these doses eliminated all clonogenic HL-60 cells.

Effect of VP-16 on Hematopoietic Colony-Forming Cells

Recovery of hematopoietic progenitors was dose and cell concentration dependent. When marrow buffy coat cells

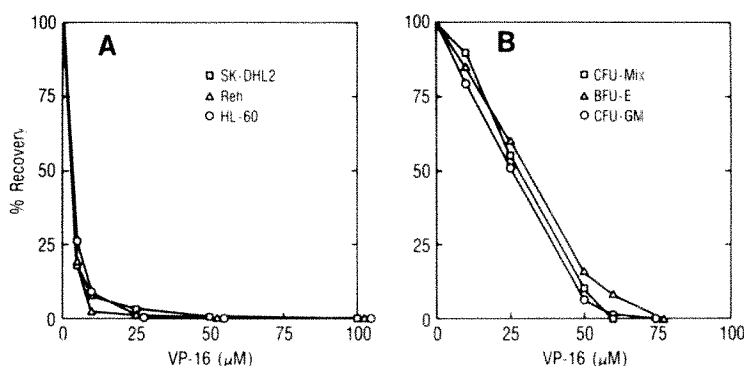


Fig 1. Comparison of the effect of VP-16 on lymphoma-leukemia cells (A) and hematopoietic progenitor cells (B). Mixtures containing 1×10^6 neoplastic cells and 2×10^6 irradiated marrow cells per milliliter were incubated with VP-16, washed, and plated for clonogenic units. Results shown are the mean of four experiments for each cell line. Controls (mean $\pm$ SEM) were: SK-DHL2, 1713 ± 554 ; Reh, 2272 ± 646 ; HL-60, 2172 ± 697 . In separate studies, normal marrow at 20×10^6 cells/mL was incubated with VP-16, washed, and assayed for colony-forming cells. Results shown are the mean (SEM $< \pm 6\%$) of four (CFU-Mix, BFU-E) and six (CFU-GM) experiments. Controls were: CFU-Mix, 10 ± 2 ; BFU-E, 69 ± 6 ; and CFU-GM, 101 ± 11 . Note the greater sensitivity to VP-16 of neoplastic cells (ID₅₀s $< 5 \mu$ mol/L) v normal hematopoietic progenitors (ID₅₀s 25 to 30 μ mol/L), and the similarity in dose-response curves of CFU-Mix, BFU-E, and CFU-GM.

Table 1. Influence of Untreated Marrow Cells on Hematopoietic Cell Growth From VP-16-Treated Human Bone Marrow

VP-16-Treated*:Untreated Ratio of Cultured Cells	CFU-Mix per 4×10^5 Cells†			BFU-E per 10^5 Cells†			CFU-GM per 10^5 Cells‡		
	Observed	Expected	O/E	Observed	Expected	O/E	Observed	Expected	O/E
3:0	1.0	—	—	7.3	—	—	8.3	—	—
2:1	3.7	4.3	0.82	23.7	26.4	0.90	36.7	39.9	0.92
1:1	7.0	6.0	1.20	34.3	36.3	0.94	53.0	56.2	0.94
1:2	7.0	7.7	0.91	47.7	46.2	1.03	71.0	72.4	0.98
0:3	11.0	—	—	65.3	—	—	104.0	—	—

O/E, observed/expected ratio.

*Marrow buffy coat cells were treated at 20×10^6 /mL with 50 μ mol/L of VP-16 for 1 hour.

†Low-density mononuclear marrow cells.

‡Marrow buffy coat cells.

were treated at 20×10^6 /mL, ID_{50} s of the growth of CFU-Mix, BFU-E, and CFU-GM were 25 to 30 μ mol/L of VP-16, and colony growth was completely abolished by 75 μ mol/L (Fig 1). In contrast, after treatment at the lower cell concentration of 5×10^6 /mL, the ID_{50} of CFU-GM was 12.5 μ mol/L, and all colony growth was inhibited by 50 μ mol/L (data not shown).

To rule out drug carryover into the culture medium or an accessory cell phenomenon, VP-16-treated marrow cells were mixed at different ratios with untreated controls and plated for CFU-Mix, BFU-E, and CFU-GM. The observed numbers of colonies were within the range expected (Table 1).

CFU-GM Production in One-Stage LTMCs

The marrow cell concentration during incubation with VP-16 significantly affected the CFU-GM production in LTMCs. LTMCs derived from marrow suspensions treated with 75 and 50 μ mol/L at 20×10^6 and 5×10^6 cells/mL, respectively—and therefore containing no CFU-Mix, BFU-E, or CFU-GM as measured by direct assay in semisolid medium—generated CFU-GM for 4 to 7 weeks (Fig 2). No progenitors were detected in either the adherent or the nonadherent fractions of LTMCs established from marrow exposed to 100 μ mol/L of VP-16.

CFU-GM production in one-stage LTMCs established from control and VP-16-treated marrow is summarized in Table 2. Mean cumulative 5-week CFU-GM yields from

LTMCs initiated with marrow incubated at 20×10^6 cells/mL and treated with 25, 50, and 75 μ mol/L of VP-16 were 60%, 18%, and 3%, respectively, of controls. Cumulative 5-week CFU-GM yields after treatments with 0, 25, 50, and 75 μ mol/L were, respectively, 127%, 153%, 290%, and a much higher but indeterminate percentage of the CFU-GM content present at the time of LTMC initiation.

LTMCs derived from marrow incubated with 25 and 50 μ mol/L of VP-16 at 5×10^6 cells/mL produced mean cumulative 5-week CFU-GM yields of 42% and 3%, respectively, of controls (Table 2). Treatments at 5×10^6 cells/mL with 0, 25, and 50 μ mol/L resulted in LTMC cumulative 5-week CFU-GM yields that were 114%, 251%, and again a higher but indeterminate percentage of the respective CFU-GM numbers of the initiating inocula.

Effect of VP-16 on Marrow Stromal Cell Proliferative Capacity

CFU-F inhibition by VP-16 was dose and cell concentration dependent (Fig 3). Increasing doses of VP-16 caused an exponential decrease in CFU-F recovery. Treatments at a high cell concentration produced less cytotoxicity: ID_{50} s of CFU-F growth were 271 and 180 μ mol/L of VP-16 after incubations at 20×10^6 cells/mL and 5×10^6 cells/mL, respectively.

Marrow treated with up to 75 μ mol/L of VP-16 and then inoculated for LTMCs gave rise to confluent stromal layers at a rate comparable to that of untreated marrow, ie, within 3

Fig 2. CFU-GM production in one-stage long-term marrow cultures (LTMCs) derived from control and VP-16-treated marrow. In these representative experiments, no corrections were made for weekly demipopulations. Note the prolonged recovery of CFU-GM from LTMCs established with marrow which at the time of LTMC initiation contained no directly measurable hematopoietic progenitors.

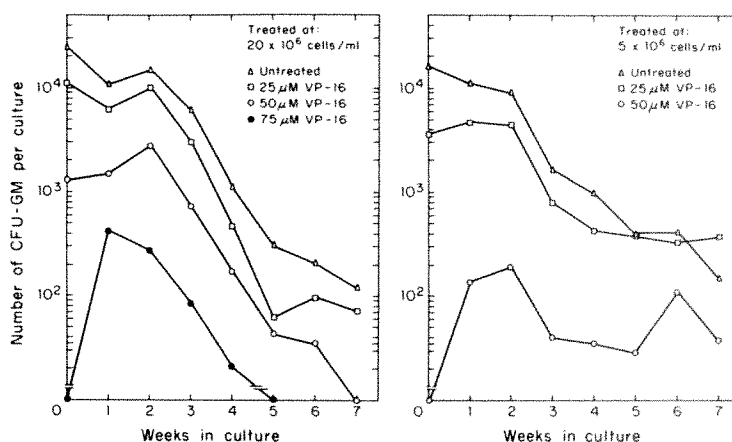


Table 2. CFU-GM Yield From One-Stage Long-term Marrow Cultures Established From Control and VP-16-Treated Marrow Suspensions

Week	Medium Control	VP-16 (μmol/L)		
		25	50	75
Treatment at 20 × 10 ⁶ cells/mL				
0 (Initial inoculum)	18,972 ± 4,870	9,470 ± 1,590	1,469 ± 156	0
1	8,545 ± 578	5,698 ± 477	1,684 ± 146	310 ± 110
2	10,990 ± 1,420	6,622 ± 817	1,945 ± 845	220 ± 55
3	3,900 ± 171	1,890 ± 759	510 ± 170	73 ± 10
4	519 ± 15	259 ± 117	102 ± 53	10 ± 5
5	86 ± 21	58 ± 12	54 ± 7	7 ± 4
Cumulative yield	24,040	14,527	4,295	620
Yield as percentage of control cultures	100%	60%	18%	3%
Yield as percentage of CFU-GM inoculum	127%	153%	292%	— *
Treatment at 5 × 10 ⁶ cells/mL				
0 (Initial inoculum)	16,675 ± 589	3,233 ± 400	0	0
1	10,833 ± 441	3,519 ± 581	234 ± 67	0
2	6,718 ± 1,483	3,590 ± 739	190 ± 5	0
3	695 ± 67	587 ± 191	25 ± 5	0
4	624 ± 41	238 ± 92	26 ± 8	0
5	203 ± 48	193 ± 40	29 ± 10	0
Cumulative yield	19,073	8,127	504	0
Yield as percentage of control cultures	100%	42%	3%	0%
Yield as percentage of CFU-GM inoculum	114%	251%	— *	0%

Mean $\pm$ SEM of four separate experiments at each treatment cell concentration. No corrections were made for weekly demipopulations. No CFU-GM were detected in cultures derived from marrow treated with 100 $\mu\text{mol/L}$ of VP-16 (data not shown).

*Because the denominator is zero, an exact figure cannot be affixed here.

to 4 weeks, and, as determined by immunofluorescence methods, contained fibroblasts, lipid-containing cells, and macrophages, with rare endothelial cells. Treatments with ≥ 100 $\mu\text{mol/L}$ of VP-16 delayed the attainment of confluence (Fig 4).

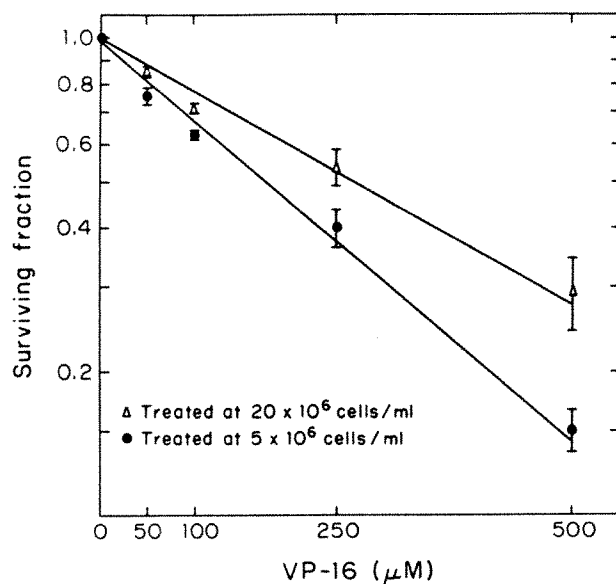


Fig 3. Survival curves for CFU-F after treatment with VP-16. Results shown are the mean $\pm$ SEM of five experiments at each cell concentration. The control CFU-F was 123 $\pm$ 9.

Functional Evaluation of Stromal Layers: CFU-GM Recovery From Two-Stage LTMCs

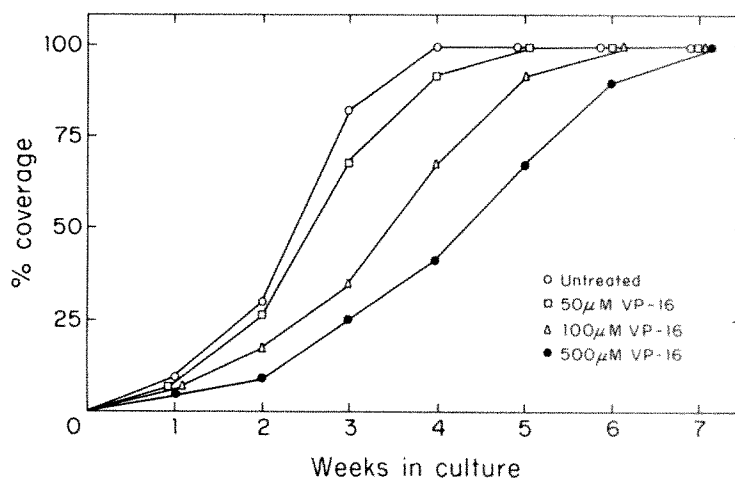
To evaluate their capacity to support hematopoiesis, confluent stromal layers were co-cultured with an autologous hematopoietic-cell-enriched marrow inoculum (NAL cells). CFU-GM recovery was comparable in two-stage LTMCs containing stromal layers derived from untreated and treated (up to 100 $\mu\text{mol/L}$ of VP-16) marrow and was greater and more prolonged than that noted when NAL cells were cultured as controls in flasks lacking stromal layers (Fig 5). CFU-GM were not detected in LTMCs to which NAL cells were not added.

DISCUSSION

Data presented herein delineate a differential sensitivity to VP-16 of normal marrow cells as compared with neoplastic cells and therefore support a role for VP-16 in marrow purging. Tumoricidal doses were defined using representative malignant lymphoid and myeloid cell lines. Neoplastic cells were treated alone or admixed with irradiated marrow cells in a simulated borderline remission situation. Treatments with 50 to 100 $\mu\text{mol/L}$ of VP-16 eliminated clonogenic malignant cells in the presence or absence of excess marrow.

After incubation of normal marrow with these doses, CFU-Mix, BFU-E, and CFU-GM were undetectable by direct assay in semisolid medium. Experiments mixing untreated and treated marrow made an accessory cell phe-

Fig 4. Kinetics of stromal layer development in long-term marrow cultures derived from control and VP-16-treated marrow. Results shown are the mean of five experiments. Stromal layers established from marrow incubated with 250 to 500 $\mu\text{mol/L}$ of VP-16 were devoid of macrophages and endothelial cells but eventually attained confluence due to proliferation of fibroblasts and lipid-containing cells.



nomenon an unlikely cause for the dose-dependent depletion of these progenitors. Colony assays have, however, been largely discredited as predictive of hematopoietic reconstitution capacity, since infusion of chemopurified marrow containing no detectable CFU-GM produces hematologic recovery in transplant patients pretreated with myeloablative chemoradiotherapy.⁶

LTCs provide an alternate means for studying hematopoietic progenitors in vitro.^{7,25-27} Increasing doses of VP-16 resulted in progressively greater LTC cumulative CFU-GM yields relative to the CFU-GM numbers present at culture initiation. Moreover, prolonged generation of CFU-GM occurred in LTCs established from marrow exposed to doses of VP-16 that abolish CFU-Mix, BFU-E, and CFU-GM in direct assays. These findings may reflect the survival of a pre-CFU-Mix population resistant to doses of VP-16 that are toxic to more mature progenitors. This interpretation presents the LTC system as a sensitive indicator of early hematopoietic precursor cell viability, as previously suggested by experiments in which marrow depleted of CFU-GM and BFU-E by complement and anti-Ia antibody generated these progenitors in LTCs.^{7,25,27} Alternatively, specific features of

the LTC system—for example, the presence of metabolically active stromal cells, or an extended time-frame that allows the repair of nonlethally injured cells or the recruitment of noncycling progenitors²⁸—may account for the detection of CFU-GM that go unidentified in semisolid medium assays. Either interpretation makes the LTC system a valuable tool for estimating hematopoietic cell survival in an ex vivo chemo-manipulated marrow.

Only very high concentrations of VP-16 (ie, 100 to 500 $\mu\text{mol/L}$) retarded the development and affected the composition of LTC adherent layers which normally contain cell types,²⁹ such as fibroblasts, lipid-containing cells, macrophages, and endothelial cells, that resemble those found in marrow studied in situ.¹¹ The largely fibroblastic nature of adherent layers derived from marrow treated with these doses of VP-16 reflects a resilience of CFU-F (ID_{50} 271 $\mu\text{mol/L}$), progenitors of the quantitatively predominant marrow stromal cell, that contrasts with hematopoietic progenitors (ID_{50} 25 $\mu\text{mol/L}$) and with clonogenic lymphoma-leukemia cells (ID_{50} <5 $\mu\text{mol/L}$). The heterogeneous adherent layers in LTCs derived from untreated marrow may be viewed as a model for the in vivo marrow microenvironment

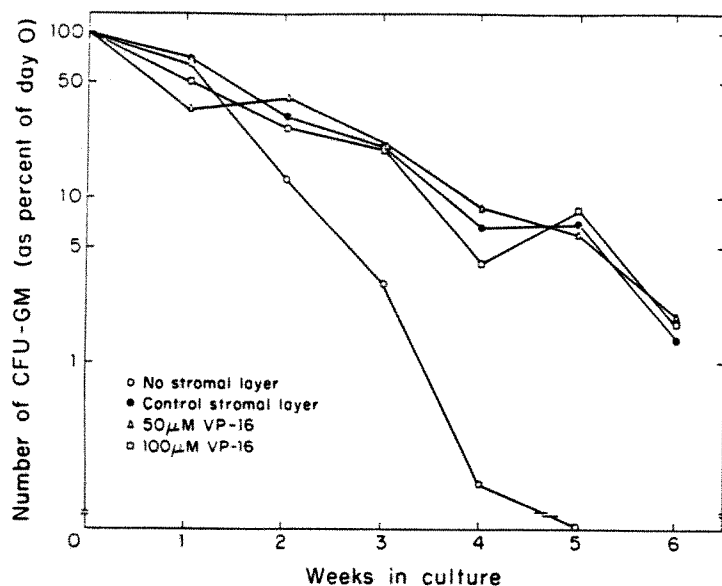


Fig 5. Hematopoietic supportive capacity of long-term marrow culture stromal layers derived from control and VP-16-treated marrow. In this representative experiment, stromal layers were seeded with NAL cells containing 16.5×10^3 CFU-GM. VP-16-treated marrow gave rise to stromal layers that sustained hematopoietic cells as well as controls. Note the sharper decline in CFU-GM in the absence of stromal layers.

because, in addition to a compositional similarity, these layers demonstrate a hematopoietic supportive capacity.²⁵ VP-16 in doses up to 100 $\mu\text{mol/L}$ did not impair this functional property, as tested in the two-stage LTMC system which showed that stromal layers established from VP-16-treated marrow can sustain hematopoietic cells as well as controls.

Although data from LTMCs encourage purging possibly contaminated marrow with doses of VP-16 higher than those defined as safe by standard myeloid progenitor cell assays, the adequacy of the antineoplastic effect of these higher doses (ie, 50 to 75 $\mu\text{mol/L}$) remains problematical. Extrapolation to the actual autotransplant setting of data generated

with cell lines requires caution since the homogeneity and the high proliferative state of the neoplastic cells, plus their lack of prior exposure to chemotherapeutic agents, promote peak tumoricidal effect. However, the greater tumor burden in the borderline remission marrow model as compared with that aimed for clinically also deserves emphasis. Resolution of this issue, as well as certitude that these doses of VP-16 are sufficiently sparing of the pluripotent stem cell compartment so as not to jeopardize posttransplant hematologic recovery, await clinical correlations. Until these points are settled, the efficacy of VP-16 against lymphoma-leukemia cells *ex vivo* might best be exploited in combination with other drugs³⁰⁻³² or with immunologically mediated purging mechanisms.³³

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Induction of NK Activity in Large Granular Lymphocyte Leukemia: Activation With Anti-CD3 Monoclonal Antibody and Interleukin 2

By Thomas P. Loughran, Jr, Kevin E. Draves, Gordon Starkebaum, Pamela Kidd, and Edward A. Clark

Large granular lymphocyte (LGL) leukemia is a rare disease characterized by clonal expansion of LGL associated with chronic neutropenia, multiple auto-antibodies, and occasionally polyarthritis. We studied cell surface antigen expression and functional activity of leukemic LGL from ten such patients. Using two-color flow cytometric analysis, we found that leukemic LGL from all ten patients expressed the CD3 and HNK-1 markers, while cells from only four patients expressed IgG Fc receptors (FcR). The LGL leukemic cells had little or no NK activity (defined as MHC-nonrestricted cytotoxicity against K562 target cells); however, NK activity could be induced in leukemic LGL by in vitro treatment with as little as 0.05 $\mu\text{g}/\text{mL}$ of anti-CD3 monoclonal antibody. Cell sorting experiments demon-

strated that NK activity was induced in CD3+ leukemic LGL (either CD3+, HNK-1+ or CD3+, FcR+) with anti-CD3 monoclonal antibody but not in normal CD3+, FcR- T cells. Treatment with purified interleukin 2 (IL 2) also caused direct activation of some CD3+ leukemic LGL. Despite induction with anti-CD3 MAb or IL 2, activated leukemic LGL did not proliferate or express high density IL 2 receptors detectable by cell sorter analysis. Treatment with alpha interferon had minimal effect on NK activity of LGL leukemic cells. These results suggest that leukemic LGL may provide a useful model for examining the signals required for LGL maturation and activation.

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A SYNDROME of chronic neutropenia associated with increased numbers of large granular lymphocytes (LGL) has been identified recently.¹ In some of these patients this disease results from a clonal expansion of LGL that is clinically associated with a variety of autoimmune phenomena.^{2,3} Some of these patients form a subgroup of Felty's syndrome.^{2,4} The CD3(T3)+, HNK-1+ phenotype of the leukemic LGL associated with low natural killer (NK) activity in vitro suggests that these cells may have arisen from normal splenic LGL.^{5,6} In other diseases studies of neoplastic cells "frozen" at stages of maturation have provided important clues concerning the differentiation of their normal counterparts.⁷⁻¹⁰ Therefore, LGL leukemia may provide a model for the analysis of LGL differentiation. In this study, we utilized two-color immunofluorescent flow cytometry analyses together with a variety of in vitro induction systems to examine maturation of leukemic LGL. Our results show that CD3+ leukemic LGL can be directly induced to acquire NK function using either anti-CD3 monoclonal antibody (MAb) or interleukin 2 (IL 2).

METHODS

Patients. Patients were referred for evaluation of chronic lymphocytosis or neutropenia; three patients had polyarthritis, five had recurrent infections. Clinical details of patients 1, 6, 9, and 10 have been previously described.^{2,4} Examination of Wright-Giemsa stained blood smears showed that all patients had markedly increased numbers of LGL (LGL counts ranged from 1,700 to 20,000 cells/ mm^3 ; normal values in our laboratory: 223 ± 99 cells/ mm^3 , $n = 10$). Chronic neutropenia (<1800 neutrophils/ mm^3) was present in all patients with the exception of patient 5 who had a normal neutrophil count. Patient 2 had acquired cyclic neutropenia. All patients were studied on protocols approved by the Human Subjects Review Committee of Fred Hutchinson Cancer Research Center or the University of Washington.

Lymphocyte phenotyping. Peripheral blood or splenic mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation and studied for expression of cell surface antigens using an EPICS 5 flow cytometer (Coulter Corporation, Hialeah, Fla) with direct one-color and two-color analysis using a panel of fluorescein-conjugated or R-phycoerythrin (PE)-conjugated mouse MAb, as previously described.¹¹ Normal human splenic tissue was obtained at time of harvesting of cadaveric kidneys for renal transplantation. The antigens recognized by the MAb are described using the current World Health Organization approved international nomenclature.¹² The following MAb have been verified by international workshop analyses: Dr Jeff Ledbetter (Oncogen Corporation, Seattle) kindly provided the G19-4 anti-CD3(T3), G17-2 anti-CD4(T4), and G10-1 anti-CD8 (T8) antibodies; Dr John Hansen (Fred Hutchinson Cancer Research Center, Seattle) kindly provided the 9.6 anti-CD2 (E-rosette receptor) and the 60.3 antibody to the Lp95-150 (LFA-1 associated) complex.¹³ Dr Toru Abo (University of Alabama, Birmingham, Ala) provided HNK-1.¹⁴ The HB10a MAb reacts with a major histocompatibility complex monomorphic determinant on Class II beta chains¹⁵ while the FC-1 MAb recognizes IgG Fc receptors (FcR; CD16) found on NK cells.¹¹ Competitive binding experiments show that FC1 recognizes an FcR epitope also recognized by Leu-11. The 3AC5 MAb recognizes a 220,000 dalton polypeptide (Lp220) of the T200 complex expressed on a subset of T helper cells.¹⁶ A high affinity (K_a approximately 10^{13} to 10^{15} mmol^{-1}) MAb 2A3 recognizing high-density IL 2 receptor (CD25) was purchased from Becton Dickinson (Mountain View, Calif) as a fluorescein conjugate.¹⁷

Cytotoxicity assay. Peripheral blood mononuclear cells were assessed for NK activity against the CD3- erythroleukemia cell line K562 using a four-hour ^{51}Cr -release assay, as previously described.¹⁸ For the sake of clear discussion, we operationally define "NK

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activity" in this paper as MHC-nonrestricted cytotoxic activity against K562. Lytic units (LU) were calculated as LU per 10^6 cells for 30% specific lysis of 10^4 target cells.

Effectors. Mononuclear cells from normals and LGL leukemic patients were isolated by Ficoll-Hypaque density centrifugation. In some experiments, mononuclear cells were enriched for LGL by a modification of the technique of Timonen and Saksela.¹⁹ In brief, nylon wool nonadherent mononuclear cells were layered over Percoll step gradients (65%/60%/55%/50%/45%/40% steps) and centrifuged for five minutes at 3,000 g at room temperature. Two-color immunofluorescent flow cytometry analyses of cells from the 50%/55% fraction showed 85% to 95% enrichment in leukemic LGL with a CD3+, HNK-1+ or CD3+, FcR+ phenotype. However, approximately 5% to 15% of cells in this fraction were CD3+, HNK-1- or CD3+, FcR-. Consequently, in some experiments two-color immunofluorescent flow cytometry was utilized to sort for CD3+, HNK-1+ or CD3+, FcR+ leukemic cells.

Induction systems. Effector cells at a concentration of 1×10^6 /mL were incubated at 37 °C in a 95% air/5% CO₂ humidified atmosphere with anti-CD3 MAb, alpha-interferon (IFN; Interferon Sciences, New Brunswick, NJ), or purified IL 2 that had been made lectin-free by chromatographic purification (Lymphocult T-LF, Biotest, Frankfurt, West Germany) for various time periods. Kinetic experiments showed that maximal induction occurred after 48 hours; therefore, subsequent experiments utilized this incubation period. Based on dose-response experiments, a dose of 1 µg/mL of anti-CD3 MAb was used in subsequent experiments. Alpha-IFN was used at a concentration of 1,000 units per 10^6 cells, whereas IL 2 was used at an optimum concentration of 1%, except in one experiment in patient 2, in which an optimum concentration of 5% was used. Other control MAb tested at a concentration of 1 µg/mL included HB10a, 3AC5 anti-Lp220, and 60.3 anti-Lp 95-150. After the incubation period, cells were washed twice, counted, and placed into the NK assay at an appropriate concentration. In addition two-color immunofluorescent flow cytometry analyses were performed after the induction period.

RESULTS

Cell surface phenotype of LGL leukemic cells. One-color analysis showed that LGL leukemic cells from all patients expressed CD2, CD3, CD5, and HNK-1 (Table 1, top). However, when other markers were examined, hetero-

geneity and unusual surface phenotypes were evident in the leukemic cells: 4 of the 10 patients (1, 3, 8, and 9) had an abnormally high percentage of cells expressing FcR (CD16), while 6 of 10 patients (3, 4, 7, 8, 9, and 10) had increased expression of HLA-DR on leukemic cells. When the leukemic cells were examined by two-color analysis (Table 1, bottom), heterogeneity in surface phenotype was even more evident. For example, many leukemic cells from patients 1, 3, and 8 expressed both CD3 and FcR, a phenotype not generally seen in normal blood lymphocytes (see below). By inference, it was evident that some CD3+, FcR+ leukemic cells from patient 3 also expressed the HLA-DR and HNK-1 markers. Leukemic LGL from patient 5 also had an unusual phenotype, coexpressing CD4 and HNK-1.

A comparison of two-color analyses of normal lymphoid tissues to leukemic cells is shown in Fig 1. Normal peripheral blood mononuclear cells had few CD3+, HNK-1+ cells and no detectable CD3+, FcR+ cells. Normal splenic cells, in contrast, had large numbers of CD3+, HNK-1+ cells; in addition, surface antigen levels of HNK-1 were higher on splenic lymphocytes than on peripheral blood mononuclear cells. The phenotype of LGL leukemic cells resembled that of normal spleen, with large numbers of CD3+, HNK-1+ cells expressing high levels of surface HNK-1. Some leukemias were CD3+, FcR+ (as illustrated by patient 1), a phenotype not readily detectable in normal peripheral blood or splenic mononuclear cells. It should be noted that leukemic cell populations contained some CD3+, HNK-1- or FcR- cells, a point discussed in more detail below.

NK activity of LGL leukemic cells. The expression of FcR and HLA-DR antigens has been associated with NK activity in normal cells,²⁰⁻²² so it was of interest that leukemic LGL from some patients expressed these markers. Previously, we found low NK activity in patients 1, 6, 9, and 10.²⁴ Table 2 shows that NK activity was also low in other patients, including patients whose cells expressed the FcR and HLA-DR markers. NK activity in patient 7 was also less than normal (data not shown); NK activity in patient 10 was not tested.

Table 1. One- and Two-Color Flow Cytometry Analyses of Peripheral Blood Mononuclear Cells From Patients With LGL Leukemia

Antigens	Patients (mean % positive cells)										Normals*
	1	2	3	4	5	6	7	8	9	10	
CD2 (E-receptor)+	59	95	98	93	79	89	99	91	83	45	71 ± 9.7
CD3 (T3)+	81	79	84	77	88	90	95	77	94	90	56 ± 6.2
CD4 (T4)+	12	27	7	12	89	44	6	43	10	12	42 ± 7.3
CD5 (T5)+	41	69	83	79	94	51	47	73	47	96	59 ± 7.3
CD8 (T8)+	37	75	71	40	7	48	54	40	58	72	22 ± 5.2
HNK-1+	65	46	57	59	77	54	54	30	44	41	13 ± 3.9
FcR (CD16)+	73	5	62	4	2	3	5	27	52	4	13 ± 6.4
HLA-DR+	6	9	54	42	3	13	85	26	26	89	11 ± 2.1
CD3+, HNK-1+	56	35	50	ND	56	50	ND	ND	ND	ND	2 ± 0.8
CD3+, FcR+	64	0	52	0	0	2	2	23	ND	ND	0
CD3+, DR+	0	2	54	41	0	5	5	12	ND	ND	0
CD8+, HNK-1+	24	41	56	21	0	33	17	20	ND	ND	3 ± 1.6
FcR+, HNK-1+	ND†	1	33	0	0	1	0	5	ND	ND	4 ± 2.4
FcR+, DR+	ND	0	54	0	0	0	0	11	ND	ND	0
CD4+, HNK-1+	ND	ND	ND	ND	58	ND	ND	ND	ND	ND	0

*Normal values shown represent mean ± standard deviation in four normal controls.

†ND, not done.

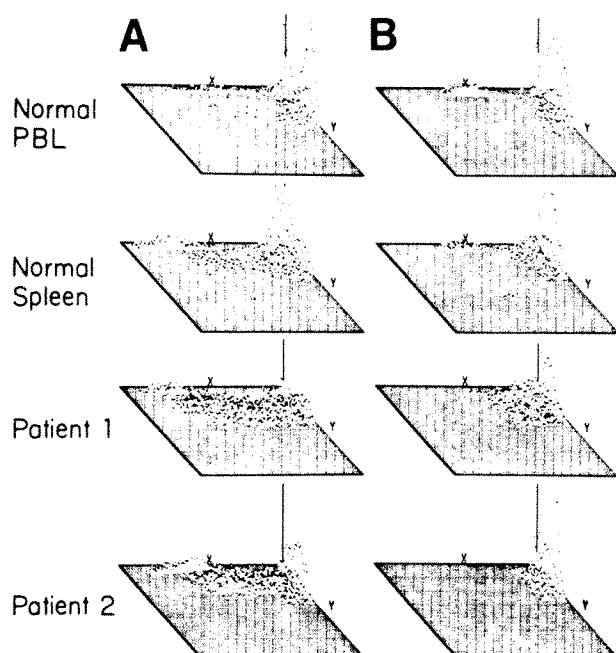


Fig 1. Two-color immunofluorescence flow cytometry analyses of mononuclear cells from normal peripheral blood and spleen, and from LGL leukemic patients utilizing (A) PE-conjugated anti-CD3 MAb (red) versus fluorescein-conjugated HNK-1 MAb (green) and (B) PE-conjugated anti-CD3 MAb (red) versus fluorescein-conjugated anti-FcR MAb (green). 64×64 grid plots represent cell numbers on vertical axis versus log of red fluorescence (Y axis) versus log of green fluorescence (X axis), with every 4 to 5 dots representing approximately a doubling of fluorescence.

However, we found that LGL leukemic cells could be induced to express NK activity by incubation with anti-CD3 MAb (Table 2). As shown in Fig 2, as little as 50 ng/mL of anti-CD3 MAb produced a dramatic increase in NK activity (uninduced: LU = 0.001; induced: LU = 1.36). The kinetics of augmentation in NK activity are shown in Fig 3. Marked increase in NK activity was observed within 24 hours; maximum induction occurred by 48 hours and subsequently waned. Addition of anti-CD3 MAb at time 0 of a four-hour assay had no effect on NK activity, also indicating that an induction period was necessary to detect optimal activity. Increased NK activity was not accompanied by any obvious increased cell proliferation as cell numbers remained con-

stant throughout these experiments. A concentration of 1 μ g/mL of anti-CD3 MAb and an incubation period of 48 hours were thus selected as optimum conditions for induction. Table 2 shows results of representative experiments utilizing this induction system on peripheral blood mononuclear cells from five LGL leukemia patients. In each experiment there was marked augmentation (9- to >10,000-fold) in NK activity. Of note, this induction was also observed in leukemic cells from patients 2, 4, and 5 which did not express FcR. Similar results were obtained when MOLT-4 cell line was used as target cells (data not shown). Similar experiments showed that anti-CD3 MAb could also increase NK activity in normal peripheral blood mononuclear cells, though this effect was generally much less dramatic (1.3- to 15-fold).

We then examined the effect of other agents on NK induction. As shown in Table 2, NK activity in normal peripheral blood mononuclear cells was also augmented by both IFN and IL 2, as previously reported.²³⁻²⁶ IL 2 increased NK activity in some LGL leukemic cells, whereas IFN had no effect. Three other monoclonal antibodies were also examined for their effect on induction (data not shown). MAb recognizing the Lp 95-150 molecular complex (60.3) decreased NK activity in the LGL leukemic cells, as has been described in normal individuals.¹³ MAb to Lp220 (3AC5) and HLA-DR (HB10a) had little or no effect on NK activity in leukemic LGL.

Since NK activity in peripheral blood mononuclear cells from LGL leukemia patients could be induced by treatment with either anti-CD3 MAb or IL 2, we examined whether leukemic LGL could be directly activated or whether induction depended on the few remaining normal T cells. Similar to previous results, anti-CD3 MAb induced NK activity in highly purified (85% to 95%) nonadherent low-density CD3+, HNK-1+ leukemic LGL from patient 1 (in two separate experiments, uninduced: LU = 0.01 and 0.01, respectively; induced: LU = 0.33 and 0.91, respectively). Because there was still a small population (5% to 15%) of CD3+ cells with a normal T cell phenotype (ie, HNK-1-, FcR-) remaining in the enriched LGL leukemic populations, it was possible that normal T cells and not the LGL leukemia cells were being induced by anti-CD3 MAb or IL 2. To exclude that possibility, two-color immunofluorescent flow cytometry was utilized to positively sort out CD3+,

Table 2. Induction of NK Activity in Peripheral Blood Mononuclear Cells From LGL Leukemia Patients

Treatment	NK Activity (LU)*							
	Patients					Normal		
	1	2	3	4	5	1	2	3†
Media only	3.7	0.01	0.07	0.30	<0.001	18.5	6.9	17.2
Anti-CD3 MAb	33.3	37.0	55.6	50.0	10.9	23.8	>100	46.5
IFN	ND‡	ND	0.03	0.20	<0.001	33.3	100.0	26.7
IL 2	ND	8.4	6.25	0.43	<0.001	14.9	33.0	33.9

*LU/ 10^6 cells for 30% specific lysis of 10^4 target cells. NK activity was assessed after 48 hours incubation with media or various agents, except in patient 2 where incubation period was 60 hours. NK activity of freshly isolated mononuclear cells from patients 1 to 5 was also assessed at time 0; results were similar to those obtained after 48 hours incubation with media alone. Anti-CD3 MAb was used at 1 μ g/mL; IFN at 1,000 U/ 10^6 cells, and IL 2 at a final concentration of 1% for all patients, except in patient 2 where IL 2 was used at concentration of 5%.

†Nylon-wool nonadherent cells.

‡ND, not done.

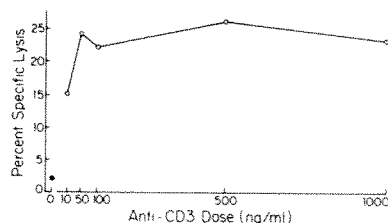


Fig 2. Dose-response characteristics of induction of NK activity in leukemic LGL by treatment with anti-CD3 MAb. Leukemic LGL were incubated with medium alone or with varying concentrations of anti-CD3 MAb for 48 hours at 37 °C in 5% CO₂ 95% air-humidified atmosphere. After incubation, cells were washed twice, counted, and used as effectors in NK assay.

FcR+ or CD3+, HNK-1+ leukemic LGL from CD3+, FcR- or HNK-1- T cells from patients 3 and 5. While CD3+, FcR+ leukemic LGL from patient 3 and CD3+, HNK-1+ (FcR-) leukemic LGL from patient 5 were directly activated by anti-CD3 MAb, no induction was seen in CD3+ T cells not expressing FcR or HNK-1 from these patients (Table 3). In patient 3, CD3+, FcR+ leukemic LGL were also directly activated by IL 2; again there was no effect of IL 2 on normal FcR- T cells from this patient.

We then examined whether induction in NK activity was associated with a change in surface antigen phenotype of the leukemic LGL. Using directly FITC-conjugated MAb and flow cytometric analysis, we found that induction with anti-CD3 MAb in leukemic LGL from patient 1 with a CD3+, HNK-1+, FcR+ phenotype did not change the density or frequency of expression of either CD3, HNK-1, FcR, or HLA-DR (data not shown). Similarly, there was no change in surface antigen expression on induced leukemic LGL from patient 5 (CD3+, HNK-1+, FcR-); in particular, there was no induction of FcR on these cells (data not shown). Augmentation of NK activity by anti-CD3 MAb or IL 2 was also not associated with expression of high-density IL 2 receptors on HNK-1+ leukemic cells (Fig 4).

DISCUSSION

The results of this study demonstrate several new findings in LGL leukemia. (1) NK activity (defined as MHC-nonrestricted cytotoxicity against K562 target cells) can be induced in leukemic LGL following incubation with either

anti-CD3 MAb or IL 2; (2) Activation with either anti-CD3 MAb or IL 2 resulted from a direct effect on CD3+ leukemic LGL and did not require CD3+ cells with a normal T cell phenotype (ie, CD3+, HNK-1-, FcR-); (3) Leukemic LGL and normal lymphocytes differed in their response to alpha IFN; and (4) Despite activation with anti-CD3 MAb or IL 2, leukemic LGL did not express detectable IL 2 receptors.

Since in other lymphoid malignancies, studies on neoplastic cells arrested at certain stages of differentiation have provided insight into the function of their normal counterparts,⁷⁻¹⁰ we examined the phenotype and function of leukemic LGL. Two-color immunofluorescent flow cytometry analyses revealed heterogeneity of phenotypes expressed on leukemic LGL. However, in all patients the majority of leukemic LGL coexpressed CD3 and HNK-1 and had little NK function in vitro. Confirming previous findings,^{5,6} we found that cells with this phenotype were present in normal spleen. Most patients with LGL leukemia have splenomegaly, and previous immunopathologic studies have shown involvement of splenic red pulp cords with leukemic CD3+, HNK-1+ cells.^{2,4} Taken together, these findings suggest a splenic origin of this disease.

Previous studies had referred to this disease as T gamma lymphoproliferative disorder, since LGL from these patients expressed CD2 and FcR.¹ Of note, cells from only four of our patients expressed FcR, thought to be a specific NK marker.^{21,22} This CD3+, HNK-1+, FcR+ phenotype of some leukemic LGL has not been readily detectable in normal fetal or adult lymphoid tissue,⁶ although peripheral blood mononuclear cells from 3 of 46 presumably normal individuals were found to have >5% circulating CD3+, FcR+ LGL.²⁷ Because of this phenotypic heterogeneity, it would seem preferable to us to refer to this disease as LGL leukemia rather than T gamma lymphoproliferative disorder.

Leukemic LGL from these patients had little NK activity in vitro, consistent with data showing that normal lymphocytes coexpressing HNK-1 and CD3 (ie, FcR-) have little cytotoxicity.¹⁹ However, functional maturation in CD3+ leukemic LGL could be consistently achieved by treatment with anti-CD3 MAb. Anti-CD3 MAb has been reported to induce cytotoxic T lymphocyte activity in normal peripheral blood mononuclear cells.^{28,29} Furthermore, induction of cytotoxic activity by anti-CD3 MAb in normal LGL has been

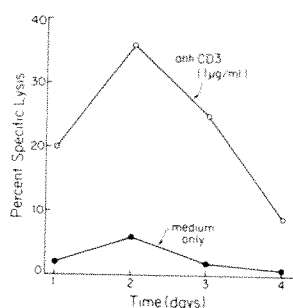


Fig 3. Kinetics of anti-CD3 MAb-induced augmentation of NK activity in leukemic LGL. Anti-CD3 was used at a concentration of 1 µg/mL; leukemic LGL were cultured at a concentration of 1×10^6 /mL. Total numbers in culture after 24 hours incubation were: 11.4×10^6 , media control, and 11.5×10^6 , anti-CD3; after 48 hours: 10.1×10^6 , media control, and 11.5×10^6 , anti-CD3; after 72 hours: 11.0×10^6 , media control, and 10.4×10^6 for anti-CD3; and after 96 hours: 9.6×10^6 , media control, and 9.2×10^6 , anti-CD3 cultures.

Table 3. Induction of NK Activity of CD3+ Leukemic LGL Purified by Cell Sorting

Cell Population	NK Activity (LU)*				
	Patient 3			Patient 5	
	Media	Anti-CD3	IL 2	Media	Anti-CD3
Unsorted	100	151	714	<0.001	0.77
CD3+ Leukemic†	143	250	294	<0.001	41.7
CD3+ Normal‡	<0.001	<0.001	<0.001	<0.001	0.008

*LU/10⁶ cells for 30% specific lysis at 10⁴ targets. NK activity was assessed after 48 hours incubation with media or various agents. Anti-CD3 MAb was used at 1 µg/mL, and IL 2 at a final concentration of 1%.

†CD3+ "leukemic" cells were obtained by two-color sorting, and defined as either CD3+, FcR+ (patient 3), or CD3+, HNK-1+ cells (patient 5). Purity of these cell populations was >97% in both cases.

‡CD3+ "normal" cells were CD3+, FcR- cells (patient 3) or CD3+, HNK-1- cells (patient 5) obtained by cell sorting.

noted, although this effect depended on normal T cells,³⁰ in contrast to our findings with leukemic LGL. The dose-response characteristics of this induction system were similar to that seen in normal LGL, with augmentation occurring with as little as 10 ng/mL of anti-CD3 and with a plateau effect seen at higher concentrations of MAb. Recently Lanier et al have induced cytotoxicity by treatment with anti-CD3 MAb in CD3+, FcR+ cells from short-term IL 2-dependent cultures derived from normal individuals.²⁷ Our findings, in addition, demonstrate that CD3+, HNK-1+ leukemic LGL that are either FcR+ or FcR- can be

directly activated by treatment with anti-CD3 MAb. However, normal T cells from these patients (CD3+, HNK-1- or CD3+, FcR-) could not be similarly activated. Taken together, these findings suggest that anti-CD3 MAb induces cytotoxicity through a direct effect on CD3+, HNK-1+ LGL.

Treatment with IL 2 also induced NK activity in leukemic LGL from 2 of 4 patients. Augmentation of NK activity by IL 2 in normal individuals has been noted previously,^{25,26} though the mechanism has not been clearly ascertained. This effect is not dependent on cell proliferation³¹ or on expression of IL 2 receptors that are recognized by the anti-TAC MAb on LGL.^{31,32} Production of gamma IFN after induction with IL 2 has been demonstrated by some investigators³¹; others have not found a contributory role for either alpha or gamma IFN in this induction.³² CD3-, IL 2 receptor- LGL were shown to respond directly to recombinant IL 2³¹; further work has shown that IL 2-responsive LGL in normal individuals are CD3-, FcR+ (HNK-1+ or -). Of note, no augmentation of NK activity was seen in peripheral blood mononuclear cells coexpressing CD3 and HNK-1³² or in CD3+, FcR+ LGL derived from IL 2-dependent short-term cultures.²⁷

The mechanism of induction of NK activity in leukemic LGL treated with IL 2 remains to be determined. Similar to findings in normal LGL, we did not find evidence for proliferation (as measured by cell counts) or expression of high-density IL 2 receptors. It is possible that alternative IL 2 receptors not recognized by the anti-IL 2 receptor may be involved in this immunoregulatory effect of IL 2.³¹ Alpha IFN does not appear to be involved in this induction system since it did not augment NK activity in leukemic LGL, unlike its effect on normal cells. In contrast to normal peripheral blood LGL,³² CD3+, HNK-1+ leukemic LGL lacking FcR could be directly activated by IL 2. Furthermore, induction of NK activity by IL 2 was also observed in CD3+, FcR+ leukemic LGL.

The activation of normal T cells and CD3+ leukemic LGL differs in that functional maturation of CD3+ leukemic LGL induced by treatment with either anti-CD3 MAb or IL 2 was not accompanied by either cell proliferation or IL 2 receptor induction, even though the anti-CD3 MAb used in this study is a potent activator of normal T cell proliferation.³³ Recently it has been shown that certain putative growth factors may instead act as differentiation factors depending on the stage of differentiation of the

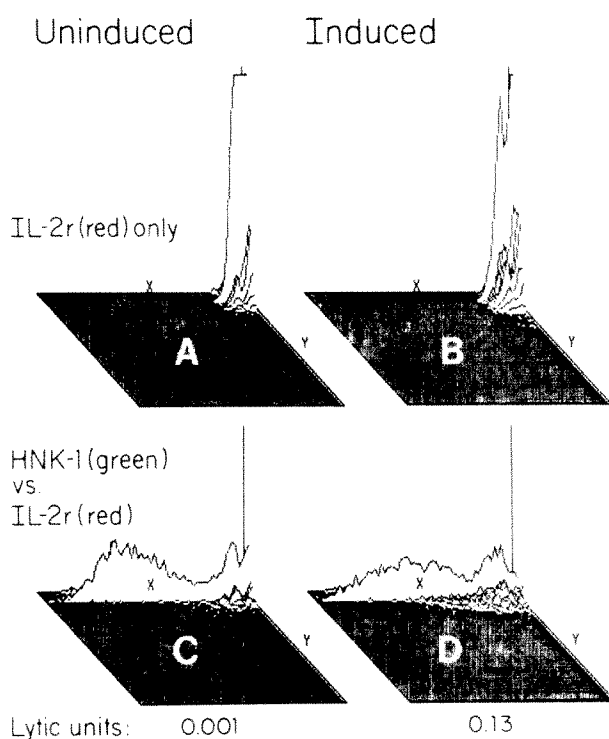


Fig 4. Lack of expression of IL 2 receptor on leukemic LGL from patient 1 even after activation with anti-CD3 MAb. One- (A and B) and two-color (C and D) flow cytometry analyses of uninduced (A and C) and induced (B and D) leukemic LGL utilized PE-conjugated anti-IL 2 receptor MAb (red) and fluorescein-conjugated HNK-1 MAb (green). 64 by 64 grid plots represent cell numbers on vertical axis versus log of red fluorescence (Y axis) versus log of green fluorescence (X axis) with every 4 to 5 dots representing approximately a doubling of fluorescence. LU/10⁶ effector cells for 30% killing of 10⁴ target cells.

induced cell.^{34,35} Our results suggest that whether or not CD3+ cells are induced to proliferate or differentiate by anti-CD3 MA b or IL 2 may depend on their state of differentiation and/or surface phenotype.

The results of these studies indicate that LGL leukemia may serve as a useful model for evaluation of LGL maturation and activation. Furthermore, these induction systems may offer an in vitro method for assessing potential therapy in these patients. The prominent autoimmune manifestations of LGL leukemia suggest an underlying defect in B-cell

immunoregulation. Since normal LGL can regulate B cells,^{36,37} the clonal expansion of functionally immature LGL may result in unopposed B cell activity. Indeed, LGL from some of these patients demonstrate impaired suppression of immunoglobulin biosynthesis, in vitro.^{2,38} Augmentation of LGL function by immunomodulatory agents therefore, may be clinically beneficial. Further studies in additional patients with LGL leukemia are needed both to assess fully the in vitro effectiveness of such agents as well as to explain their mechanism of action.

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Immunoglobulin and T Cell Receptor Gene Rearrangements in Human Lymphoma and Leukemia

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DNA samples from blood leukocytes or tumor biopsies of 45 patients with phenotypic B or T cell neoplasms were analyzed for rearrangements of the immunoglobulin (Ig) or T cell receptor (TCR) genes by Southern blot hybridization analysis. Rearrangements of the Ig heavy chain joining region genes (J_H) were present in DNA from each of 28 B cell lymphomas and leukemias; 14 of 21 of these tumors also had rearrangements of the Ig kappa light chain joining (J_K) or deleting element (K_{del}) genes. Conversely, 16 of 17 T cell lymphomas and leukemias had rearranged TCR beta chain genes. One B cell and one T cell tumor had rearrangements of both Ig and TCR genes. There was a strong correlation between the rearrangements of specific genes

and the immunophenotype of the tumor: (1) J_H rearrangement without TCR beta chain rearrangement occurred only in B cell tumors; (2) TCR beta chain rearrangement with or without J_H rearrangement occurred only in T cell tumors, with one exception; and (3) J_K and K_{del} rearrangements were found only in B cell tumors. Thus, rearrangements of the Ig heavy and light chain genes and the TCR beta chain genes were found to be highly sensitive markers of monoclonal human lymphomas and lymphoid leukemias, with the type of gene rearrangements well correlated with the cell lineage of these neoplasms.

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NORMAL B and T lymphocytes can express a virtually unlimited repertoire of antibody and T cell receptor specificities. Such diversity is made possible by variations in the assembly of the immunoglobulin and T cell receptor genes.¹⁻¹² Discontinuous genes encoding the variable regions of immunoglobulin (Ig) heavy and light chains undergo somatic DNA rearrangement during the differentiation of lymphoid stem cells into B lymphocytes.^{1,2} Similarly, rearrangements of the T cell receptor (TCR) alpha and beta chain genes occur during the early stages of T lymphocyte differentiation.³⁻⁶ Because of the large number of possible gene rearrangements, the structure of Ig or TCR genes is virtually unique for each B or T lymphocyte.

Southern blot assays of DNA from human lymphoid neoplasms have shown that the tumor cells of individual neoplasms generally contain a single pattern of Ig or TCR gene rearrangement, with variations in the structure of these rearrangements from tumor to tumor.¹³⁻¹⁷ These findings confirmed the monoclonal origin of these tumors and showed that Southern blot assays can detect clonal proliferations of lymphocytes which comprise as little as 1% to 5% of the total cells in the tissue sample.¹⁴ The DNA from polyclonal proliferations of lymphocytes do not contain detectable specific gene rearrangements because the structure of the rearranged gene(s) varies from cell to cell.^{15,16}

One advantage of gene rearrangement assays is that the monoclonality of lymphoproliferative lesions can be established in the absence of the expression of cellular phenotypic markers, such as the monotypic expression of Ig kappa or lambda light chains. Assays for gene rearrangements have been used to establish the B cell origin of certain human lymphoid tumors. Thus, "non-T, non-B" acute lymphoblastic leukemia,^{13,18} hairy cell leukemia,¹⁹ lymphoid blast crises of chronic myelogenous leukemia,^{20,21} and certain lymphomas¹⁵ were shown to arise from B lymphocytes by virtue of rearranged Ig heavy and light chain genes. However, Ig heavy chain gene rearrangements are not totally specific for B cells, since they have been described in T cell leukemia,²² acute nonlymphocytic leukemia,²³ and in some murine T cell lymphomas and cultured T cell lines.^{24,25} TCR beta chain genes (T_{beta}) have been shown to rearrange in T cell lines and

in some human T cell tumors, but the specificity of such rearrangements has not yet been fully defined.^{10,11,17,26}

The purpose of the present study was to compare the frequency and pattern of Ig and T_{beta} gene rearrangements in a wide variety of histopathologically and immunophenotypically defined human lymphomas and leukemias.

MATERIALS AND METHODS

Patients. Forty-five patients with lymphoma or lymphoid leukemia were included in the study, selected on the basis of tumor morphology and/or immunophenotype. This investigation was approved by the Human Investigations Committee of the University of Virginia.

Tissue accrual and storage. Tissue samples were obtained from excess material from biopsies or blood samples obtained for diagnostic purposes. After submitting necessary tissue for histopathologic and immunocytochemical studies, the remaining tissue or blood cells were snap-frozen in liquid nitrogen or a dry-ice-methanol bath and stored at -70°C or in liquid nitrogen vapors.

Morphologic classification. Standard morphologic criteria for the diagnosis and subclassification of acute and chronic lymphocytic leukemias were used, based upon Wright's stain and cytochemical studies of blood or bone marrow cells with periodic acid-Schiff reagent (PAS), myeloperoxidase, nonspecific esterase (NSE), and Oil Red O.^{27,28}

Non-Hodgkin's lymphomas were classified by the New Working Formulation of the National Cancer Institute.²⁹ Plastic or paraffin-

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embedded biopsy samples were stained with hematoxylin and eosin for interpretation.

Immunocytochemical phenotyping. The cellular phenotype of tumor samples was determined by immunoperoxidase staining of tissue sections or by analysis of cell suspensions by indirect immunofluorescence or immunoperoxidase staining or by flow cytometry.^{30,31} At least 25% of tumor cells were required to express an antigen in order for the tumor to be considered positive for that marker. Tumors were classified as B cell in origin if the tumor cells were positive for the presence of surface or cytoplasmic Ig and/or for the monoclonal antibodies Leu 12, Leu 14, B1, or B2. Tumors were classified as T cell in origin if the cells expressed no B cell markers and expressed one or more of the pan T cell markers Leu 1, Leu 4, Leu 5, and Leu 9. T cell subset markers including markers for T helper cells (Leu 3A, OKT4), T suppressor cells (Leu 2A, OKT8), thymic cells (Leu 6, OKT6), and natural killer cells (Leu 7, Leu 11) were assayed.

The nonspecific cellular markers HLA-Dr (Ia-like), common acute lymphocytic leukemia antigen (CALLA, J5), and terminal deoxynucleotidyl transferase enzyme (Tdt) were also assayed in some cases.

Immunoglobulin gene rearrangement. DNA was isolated from tissue samples by standard phenol-chloroform extraction and ethanol precipitation.³² Five micrograms of DNA from each sample were digested overnight with 5 to 10 U/mcg of the restriction enzyme Bam H1 or Eco R1 (Bethesda Research Laboratories, Inc, Gaithersburg, MD). The resulting DNA fragments were separated by electrophoresis on 0.6% agarose gels, and the DNA was transferred to a nylon membrane (Zeta Probe, BioRad Laboratories, Richmond, Calif) by the method of Southern.³³ Membrane-bound DNA was then hybridized with a nick translated probe³⁴ from the Ig heavy chain joining region (J_H). The J_H probe, a 6 Kb Bam H1-Hind III genomic fragment containing the J_H region, was cloned into the plasmid pBR 322 (kindly provided by Dr Philip Leder, Harvard University, Boston). The probe was gel purified from plasmid DNA prior to nick translation. Specific activity of the ^{32} P-labelled J_H probe was 1.5 to 3.0×10^8 cpm/mcg DNA. Hybridization was carried out at 65°C for 18 to 22 hours followed by high stringency washing of the blot. Membranes were exposed to Kodak XRP-5 film at -70°C for 1 to 10 days with intensifying screens. The blots were then stripped in 0.4 N NaOH , neutralized, and stored in prehybridization mix prior to rehybridizing with another probe.

Kappa light chain gene rearrangements were detected in Bam H1 genomic DNA digests using two probes. A 4.6 Kb Hind III fragment

containing the kappa joining region genes (J_K) was subcloned in pBR 322 from a genomic kappa constant and joining gene probe (provided by Dr Philip Leder). A 2.5 Kb Bam H1-Hind III fragment containing the kappa deleting element (K_{Del}) was cloned in pBR 322 (kindly provided by Dr Stanley Korsmeyer, National Institutes of Health³⁵).

Rearrangements of the Ig genes were detected by the appearance of non-germline DNA band(s) hybridizing to the ^{32} P-labelled J_H probe (Fig 1) and the appearance of new band(s) and/or deletion of the germline band with the J_K probe (Fig 1). The K_{Del} genes are located 3' of the kappa light chain constant region (C_K) locus on chromosome 2p, and are postulated to mediate deletion of C_K and J_K following unsuccessful rearrangement of a kappa allele.³⁵ Thus, a tumor with successful rearrangement of the first kappa allele should show germline K_{Del} genes. A tumor expressing kappa light chains from rearrangement of the second allele (the first being nonfunctionally rearranged) usually contains two germline K_{Del} genes, although K_{Del} rearrangement may be seen in the nonfunctional allele if C_K has been deleted. If both kappa alleles are deleted, as in some lambda producing tumor cells, both K_{Del} genes should be rearranged. Autoradiograph patterns of K_{Del} rearrangements are shown in Fig 2.

T cell receptor gene rearrangement. T_{beta} gene rearrangements were assayed as described above for the Ig genes, using Bam H1, Eco R1, and Hind III restriction enzyme digests of tumor DNA. The probe was a cDNA clone of the TCR beta chain, containing both variable and constant regions (kindly provided by Dr Tak Mak, University of Toronto). This 770 base pair probe was cloned into the Pst I site of pBR 322, and was gel purified from plasmid DNA prior to nick translation. In some cases hybridization was performed using a 440 base pair constant region probe obtained by Hinc II restriction enzyme cleavage of the intact cDNA, with purification of the constant region fragment by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Criteria for rearrangement of T_{beta} genes included the appearance of new bands on the autoradiographs of tumor DNA plus the partial or complete deletion of constant region germline bands (Fig 3).

RESULTS

A total of 45 patients were included in this study. In some cases serial biopsies or multiple simultaneously obtained tissue samples were available. In all cases the extracted DNA from the frozen specimens was well preserved, with little or no degradation.

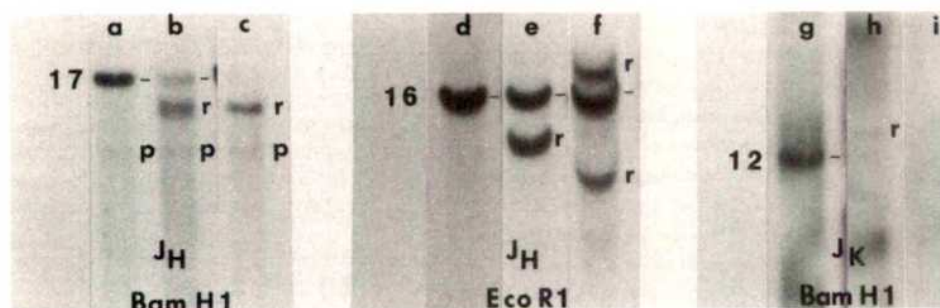


Fig 1. Rearrangement patterns of immunoglobulin heavy chain joining region (J_H , lanes a–f) and kappa light chain joining region (J_K , lanes g–i) genes on Eco R1 and Bam H1 digests of tumor DNA. Germline bands (dash marks, lanes a, d, g), rearranged bands (r), and pseudogene bands (p) plus the germline band size in kilobase units are indicated. Lane a, control DNA. Lane b, partial deletion of germline J_H band, one rearranged allele (case no. 85-8, Table 2). Lane c, one rearranged and one deleted J_H allele (case no. 85-12, Table 1). Lane d, control DNA. Lane e, one rearranged J_H allele (case no. 83-8, Table 1). Lane f, both J_H alleles rearranged, residual germline band from nontumor cell DNA (case no. 84-16, Table 2). Lane g, control DNA. Lane h, one rearranged and one deleted J_K allele (case no. 83-12, Table 1). Lane i, deletion of both J_K alleles (case no. 83-2, Table 2). See restriction enzyme maps, reference 15.

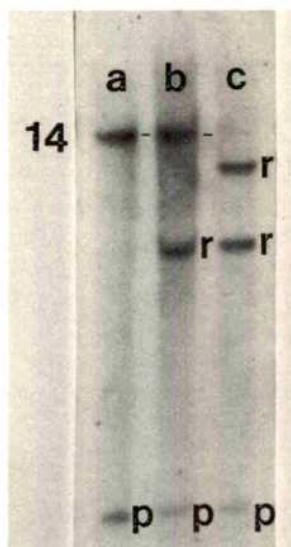


Fig 2. Rearrangement patterns of immunoglobulin kappa light chain deleting element (K_{Del}). Lane a, control DNA; germline pattern with 14Kb germline band (dash mark) and approximately 3Kb pseudogene (p). Lane b, one rearranged K_{Del} allele (case no. 84-3, Table 2). Lane c, both K_{Del} alleles rearranged (case no. 83-12, Table 1). DNA was digested with Bam H1. See reference 35.

Lymphoid leukemia. Nine cases of acute lymphoblastic leukemia (ALL) were evaluated. The DNA was extracted from bone marrow cells in six cases, peripheral blood cells in two cases, and from a testicular biopsy in one case (case no. 85-20). The tumor cell phenotypes and results of the gene

rearrangement assays are presented in Table 1. Three cases of "pre-B cell" ALL showed rearrangement of Ig heavy chain alleles by J_H probe hybridization. Two showed germline T_{beta} genes; the remaining case could not be studied for the T_{beta} marker due to inadequate material.

Six T cell ALLs were evaluated; one was HLA-Dr positive and four were J5 positive. Cells from all six were found to have T_{beta} gene rearrangement; one (case no. 83-8) also had rearrangement of one Ig J_H allele by Eco R1 (Fig 1, Lane e) but not Bam H1 restriction enzyme digestion of the tumor DNA. J_K and K_{Del} gene rearrangements were not present in the five T cell ALLs tested, including the tumor with J_H rearrangement. Detection of J_H gene rearrangements on Eco R1 but not Bam H1 digests has been noted previously in ALL.¹³

Two cases of hairy cell leukemia had Ig gene rearrangement without TCR gene rearrangement (Table 1), consistent with the B cell lineage previously reported for this neoplasm.¹⁹

B cell lymphomas. Twenty-three phenotypic B cell lymphomas were studied (Table 2). Eighteen of 19 tumors studied for surface or cytoplasmic Ig were positive, as were all 14 tumors tested with B1, B2, Leu 12, and/or Leu 14 monoclonal antibodies. Two follicular mixed small cleaved and large cell lymphomas did not have phenotypic marker studies performed (case nos. 83-2 and 85-23), but were included in this category because virtually all true follicular lymphomas are B cell in origin.^{36,37}

Each of the 23 B cell lymphomas had rearrangement of one or both Ig J_H alleles (Table 2). Six of seven tumors had J_K rearrangements and/or deletions, while 12 of 17 had K_{Del} rearrangements. One diffuse large noncleaved cell lym-

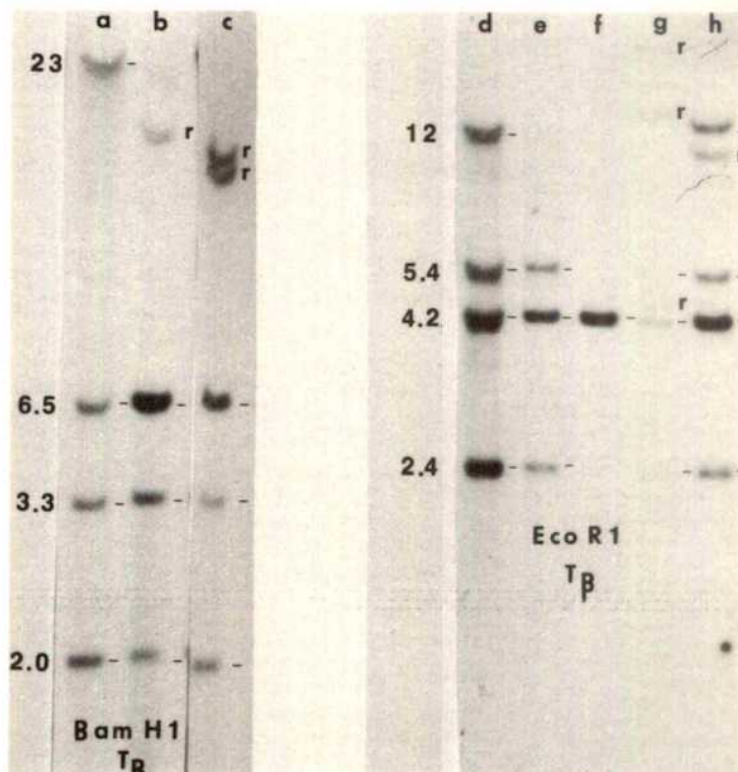


Fig 3. Rearrangement patterns of T cell receptor beta chain genes (T_{beta}) on Bam H1 and Eco R1 digests of tumor DNA. The T_{beta} probe was from a cDNA clone containing both constant region (23Kb band Bam H1, 12 and 4.2Kb bands Eco R1) and variable region genes (2.0, 3.3, and 6.5Kb bands Bam H1; 2.4 and 5.4Kb bands Eco R1). Lanes a and d, control DNA; germline patterns (dash marks). Lane b, one rearranged (r), one deleted T_{beta} constant region allele (case no. 84-5, Table 1). Lane c, both constant region alleles rearranged (case no. 83-8, Table 1). Lane e, deletion of first constant region alleles (case no. 83-8). Lane f, deletion of first constant region alleles and both variable region bands (T cell line HPB-MLT). Lane g, deletion of first constant region alleles, three non-germline bands representing rearranged variable and/or constant region genes (T cell line HSB). Lane h, one rearranged band (T cell line JM). See restriction enzyme maps, references 43, 47.

Table 1. Lymphoid Leukemias

Case No.	Phenotypic Markers*												Gene Rearrangements			
	Nonspecific			B Cell		T Cell										
	HLA-Dr	J5 (CALLa)	Tdt	Ig	Leu 12 Leu 14	Leu 5	Leu 1	Leu 9	Leu 4	Leu 3A	Leu 2A	Leu 6	J _H	J _K	K _{Del}	T _β
Pre-B cell ALL																
85-12	+	+	NA	NA	+	—	—	—	—	—	—	—	1R, 1D	GL	GL	GL
85-21	+	+	NA	cM	+	—	—	—	—	—	—	NA	1R, 1D	NA	NA	NA
85-60	+	—	+	—	+	NA	—	NA	—	—	—	NA	1R, 1GL	NA	1R, 1GL	GL
T cell ALL																
83-8	+	+	+	—	NA	+	+	+	+	+	+	+	1R, 1GL	GL	GL	2R
83-17	—	+	NA	—	NA	—	—	+	+	+	NA	NA	GL	GL	NA	2R
84-5	—	—	+	—	NA	—	NA	+	NA	—	—	—	GL	NA	NA	1R, 1D
84-29	—	—	NA	NA	NA	—	+	+	NA	—	—	—	GL	GL	GL	2R
85-19	—	+	NA	NA	NA	—	+	+	—	—	—	NA	GL	GL	GL	2R
85-20	—	+	+	NA	—	+	+	+	+	+	—	+	GL	GL	GL	2R
Hairy cell leukemia																
83-12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1R, 1D	1R, 1D	2R	GL
85-104	NA	NA	NA	M, D _κ	NA	—	NA	NA	—	—	—	NA	1R, 1GL	NA	GL	GL

Abbreviations: R, Rearranged; GL, Germine; D, Deleted; cM, cytoplasmic IgM.

*See text for marker definitions; Leu monoclonal antibodies or equivalent OK-T antibodies were used. NA, not available; "—" ≥25% of tumor cells reacting with a monoclonal antibody.

Table 2. B Cell Lymphomas

Case No.	Lymphoma Histology	Phenotypic Markers*						Gene Rearrangements			
		J5 (CALLa)		B1 B2		Leu 12 Leu 14					
		HLA-Dr	Ig	Ig	Ig	Ig	Ig	J _H	J _K	K _{Del}	T _β
83-78	Small noncleaved cell	+	+	M _κ	NA	+	+	1R, 1GL	1D, 1R	1R, 1GL	GL
83-10	Small noncleaved cell	+	+	D _λ	NA	+	+	1R, 1GL	NA	NA	GL
84-18A	Lymphoblastic	+	—	cM	NA	+	+	1R, 1GL	GL	GL	GL
84-16	Diffuse large cell, immunoblastic clear cell	NA	NA	NA	+	+	+	2R	NA	2R	GL
84-20A	Diffuse large cell, immunoblastic plasmacytoid	NA	NA	κ	+	+	+	2R	NA	GL	GL
83-2	Follicular mixed small cleaved & large cell	NA	NA	NA	NA	NA	NA	1R, 1GL	NA	NA	GL
83-11	Follicular mixed small cleaved & large cell	+	+	G _κ	+	NA	+	1R, 1GL	NA	GL	GL
84-2	Follicular mixed small cleaved & large cell	NA	—	M _κ	NA	NA	NA	1R, 1GL	NA	NA	GL
84-3	Small lymphocytic	NA	NA	G _κ	NA	NA	NA	1R, 1GL	1R, 1GL	1R, 1GL	GL
84-4	Diffuse large noncleaved cell	+	NA	κ	NA	+	+	1R, 1GL	NA	NA	GL
84-15C	Diffuse large cell immunoblastic plasmacytoid	NA	NA	λ	NA	NA	NA	1R, 1GL	NA	2R	GL
85-18	Diffuse large noncleaved cell	+	+	M _λ	+	+	+	1R, 1D	1R, 1GL	1R, 1GL†	GL†
84-11	Small noncleaved cell (Burkitt's)	+	—	M _κ	NA	+	+	2R	NA	1R, 1GL	1R, 1D
84-22A	Follicular small cleaved cell	NA	+	NA	+	+	+	1R, 1GL	D	2R	GL
85-23	Follicular mixed small cleaved & large cell, with diffuse areas	NA	NA	NA	NA	NA	NA	2R	NA	1R, 1GL	GL
85-8	Small lymphocytic (intermediate)	NA	NA	λ	NA	+	+	1R, 1D	D	2R	GL
85-28	Small lymphocytic (intermediate)	NA	NA	κ	+	+	+	2R	2R	1R, 1GL	GL
85-68	Follicular & diffuse small cleaved cell	NA	NA	M, D _κ	NA	NA	NA	1R, 1GL	NA	NA	GL
85-75	Diffuse large noncleaved cell	NA	NA	M, D _κ	NA	NA	NA	2R	NA	GL	GL
85-97	Follicular small cleaved cell, with diffuse areas	+	NA	—	+	NA	NA	1R, 1GL	NA	2R	GL
85-105	Diffuse noncleaved large cell	NA	NA	D _κ	NA	NA	NA	1R, 1GL	NA	1R, 1GL	GL
85-108	Diffuse small cleaved cell	NA	NA	M, D _λ	NA	NA	NA	1R, 1GL	NA	NA	GL
85-113A	Small lymphocytic, plasmacytoid (Waldenström's macroglobulinemia)	NA	NA	κ	+	+	+	2R	NA	GL	GL

Abbreviations: NA, Not Available; "—" ≥25% of tumor cells reacting with a monoclonal antibody; R, Rearranged; GL, Germine; D, Deleted; κ, Kappa light chain; λ, Lambda light chain; M, IgM; cM, cytoplasmic IgM; G, IgG; D, IgD.

*See text for marker definitions; Leu monoclonal antibodies or equivalent OK-T antibodies were used.

†See text for discussion.

phoma positive for surface IgM lambda (case no. 85-18) did not show the predicted pattern of having both kappa alleles rearranged.^{16,18} Instead one germline and one rearranged band were present with both J_K and K_{Del} probes. It is unlikely that these bands represent rearranged genes comigrating in the germline position because the germline fragments for J_K and K_{Del} are of different size (12 kb and 14 kb, respectively). This pattern of gene rearrangement is unexpected since B cells normally attempt to rearrange both kappa alleles before proceeding to lambda light chain rearrangement.^{16,18} However, a similar exception to this normal hierarchical Ig gene rearrangement was recently reported for a lambda-producing human lymphoid cell line.³⁸

One B cell lymphoma had detectable T_{beta} gene rearrangement on both Bam H1 and Eco R1 digests of tumor DNA, in addition to rearrangements of J_H and K_{Del} (case no. 84-11). This small noncleaved cell (Burkitt's) lymphoma stained positive with Oil-Red-O but negative with myeloperoxidase, NSE, and PAS. The tumor cells expressed cell surface IgM_K, HLA-Dr, and Leu 12, but they failed to react with antibodies to J5, Leu 1, Leu 4, and Leu 9 and did not form E rosettes.

T cell lymphomas. Eleven T cell lymphomas were evaluated (Table 3). A variety of histologic types were represented, and all were negative for surface or cytoplasmic Ig and B cell monoclonal antibody markers. Most tumors were positive for the pan-T cell markers detected by the Leu 5, Leu 9, or Leu 4 antibodies. Six tumors expressed Leu 3A (T helper cells), one expressed Leu 2A (T-suppressor cells), one expressed both Leu 3A and Leu 2A, and three were negative for both markers.

Ten of the eleven tumors had rearranged T_{beta} genes. The one tumor in germline configuration was a lymphoblastic lymphoma which was Leu 1, 5, and 9 positive, but negative for the Leu 4 monoclonal antibody and the Leu 2A and 3A subset markers (case no. 84-6). The lack of expression of mature T cell markers, including the TCR complex detected by the Leu 4 antibody, suggested that this neoplasm has arisen from a primitive T cell. The absence of detectable T_{beta} gene rearrangements may have been due to the immaturity of the tumor cells.

No Ig gene rearrangements were seen in these phenotypic T cell lymphomas.

Analysis of DNA from normal tissues and other lymphoproliferative states. DNA was extracted from peripheral blood cells or cultured skin fibroblasts from 27 normal human donors. Germline T_{beta} genes were present in all cases using Bam H1, Eco R1, and Hind III restriction enzyme DNA digests. No K_{Del} polymorphisms were seen in 15 normal DNA samples digested with Bam H1, Eco R1, or Hind III.

Tissues from several other lymphoproliferative diseases were also available for study (data not shown). The DNAs from a dermatopathic lymph node, from lymph nodes of three patients with acquired immune deficiency syndrome (AIDS)-associated lymphadenopathy, and biopsies from two cases of thymic hyperplasia lacked Ig or T_{beta} gene rearrangements. Interestingly, analysis of DNAs from both the thymus samples showed a decreased intensity in the bands representing the unrearranged TCR beta chain constant region genes. This was most evident in the partial deletion of the 12Kb Eco R1 germline band, which indicated that a substantial proportion of the thymocytes contained rearrangements of the first constant region locus of the T_{beta} genes.¹⁷ The absence of any detectable rearranged band in the autoradiographs revealed the polyclonal nature of this process. No rearrangements were present in six cases of Hodgkin's lymphoma (four nodular sclerosis, one mixed cellularity, and one lymphocyte predominant histology).

DISCUSSION

Rearrangements of the Ig and TCR genes were found to be highly sensitive markers for a wide spectrum of histopathologic and immunologic phenotypes of B and T cell neoplasms. Southern blot assays for somatic rearrangements of the J_H region of the Ig heavy chain gene, the J_K and K_{Del} regions of the Ig kappa light chain genes, and the TCR beta chain gene were positive in 44 of the 45 lymphoid leukemias and lymphomas that were studied. These observations are in agreement with the concept that Ig or TCR gene rearrangements occur early in the differentiation of B or T lymphocytes and that the vast majority of human lymphoid neoplasms are monoclonal in origin.^{13,18,39,40}

There was a strong correlation between the rearrangements of specific gene regions in the cellular DNA and the

Table 3. T Cell Lymphomas

Case No.	Histology	Phenotypic Markers*													Gene Rearrangements			
		Nonspecific		B Cell			T Cell											
		HLA-DR	J5 (CALLa)	Ig	B1 B2	Leu 12 Leu 14	Leu 5	Leu 1	Leu 9	Leu 4	Leu 3A	Leu 2A	Leu 6	Leu 11				
															J _H	J _K	K _{Del}	T _β
84-6	Lymphoblastic	—	—	—	NA	—	+	+	+	—	—	—	NA	NA	GL	NA	NA	GL
84-27	Lymphoblastic	—	—	—	NA	NA	+	+	+	NA	+	—	—	NA	GL	GL	GL	1R, 1D
84-19A	Cutaneous (small lymphocytic)	+	NA	—	NA	—	+	+	+	+	+	+	—	—	GL	NA	GL	1R, 1D
84-28	Cutaneous (large cell)	+	NA	NA	NA	NA	+	NA	—	+	—	—	NA	NA	GL	GL	GL	1R, 1GL
85-29C	Cutaneous (small lymphocytic)	NA	—	NA	—	—	+	—	+	+	—	—	NA	NA	GL	NA	GL	1R, 1D
85-77	Cutaneous (large cell)	NA	NA	NA	—	—	+	+	—	+	+	—	—	NA	GL	NA	GL	1R, 1D
85-93	Mycosis fungoides	NA	NA	—	NA	—	+	NA	NA	NA	+	—	NA	NA	GL	NA	GL	1R, 1GL
85-117	Mycosis fungoides	—	—	NA	NA	—	+	+	—	+	+	—	—	—	GL	NA	GL	1R, 1D
84-25	Diffuse large cell	+	NA	—	NA	NA	+	+	+	+	—	+	NA	NA	GL	GL	NA	1R, 1D
84-26	Small lymphocytic	+	—	—	NA	NA	+	+	+	NA	+	—	NA	NA	GL	GL	NA	2R
85-88	Diffuse small cleaved cell	—	NA	NA	—	NA	+	NA	NA	NA	—	—	NA	NA	GL	NA	GL	2R

Abbreviations: R, Rearranged; GL, Germline; D, Deleted.

*See text for marker definitions; Leu monoclonal antibodies or equivalent OK-T antibodies were used. NA, Not Available; "+" ≥ 25% of tumor cells reacting with a monoclonal antibody.

immunophenotype of individual tumors. First, J_H rearrangement without T_{β} rearrangement occurred only in B cell tumors. Second, T_{β} rearrangements with or without J_H rearrangement occurred only in T cell tumors with one exception. Third, J_K and K_{Del} rearrangements were highly specific for B cell tumors. Thus, based on J_H and T_{β} gene rearrangements, one could correctly predict whether a given lymphoid tumor was of B or T cell phenotype in 42/45 cases (93%). In this series assays for rearrangement of the J_K and K_{Del} genes established the tumor phenotype in one case with coexisting J_H and T_{β} rearrangements, but otherwise did not improve the ability to predict tumor phenotype.

The DNA from three tumors showed atypical patterns of Ig and TCR gene rearrangements. One lymphoblastic lymphoma of T cell phenotype had no detectable gene rearrangements (case no 84-6, Table 3). This may represent a false-negative assay in which the DNA fragments from the rearranged allele comigrated with those genes in the germline configuration, although this was unlikely to have occurred in all restriction enzyme digests. Alternatively, the failure of these cells to express the Leu 4 molecule, an integral part of the T cell receptor,⁴¹ may have reflected the absence of T_{β} gene rearrangements and production of a functional TCR beta chain. The phenotype of the lymphoma cells was similar to that of a subset of normal immature thymocytes that may not contain rearranged TCR genes.^{39,40} One T cell ALL contained rearrangement of the T_{β} genes and of the Ig J_H region (case no. 83-8, Table 1). Rearrangement of Ig mu-chain genes has been described in T cell ALL.²² J_H rearrangement in T cell ALL may be analogous to rearrangements in some murine T cell lymphomas in which a fusion of Ig heavy chain diversity genes (D_H) and J_H segments has occurred.^{24,25} In Southern blot assays these fusions appear as rearrangements of the Ig J_H genes. However, the variable region sequences are absent and these rearranged genes are incapable of encoding functional Ig heavy chain molecules.

The third atypical tumor contained rearrangements in each of the three gene regions that were analyzed. This small noncleaved cell (Burkitt's) lymphoma (no. 84-11, Table 2) expressed cell surface IgM κ , with no evidence of a coexisting T cell clone to account for the T_{β} rearrangement. Others have also found so-called bigenotypic rearrangements in up to 11% of phenotypic B and T cell tumors.^{42,43} O'Connor et al²⁶ reported that Ig heavy chain and T_{β} rearrangements coexisted in three of 52 cases of B cell chronic lymphocytic leukemia and lymphoma. Pelicci et al⁴² found such dual rearrangements in five of 33 B cell and in two of 30 T cell malignancies. The frequency of such nonlineage specific rearrangements will be an important consideration when attempting to use Ig and TCR gene rearrangement assays to assign the cell lineage of phenotypically undefined lymphoid neoplasms.

This and other recent reports have underscored the usefulness of assays for T_{β} gene rearrangements as the first reliable method for establishing monoclonality in T cell proliferations.^{17,26,43-47} Although T cell lymphomas may display abnormalities in the expression of certain T cell differ-

entiation antigens, this finding is not invariable and only indirectly infers monoclonality.⁴⁸ T_{β} gene rearrangements were highly sensitive markers of T cell neoplasms and were present in 16 of our 17 T cell cases and in nearly all T cell leukemias and lymphomas reported by others.^{17,26,42-47} Important in this regard is the apparent low frequency of polymorphisms for the T_{β} genes, occurring in none of our 27 normal human DNA samples. However, rare polymorphisms at the Eco R1 or Hind III sites have been noted by others.^{17,43}

Deletion of the first TCR beta chain constant region (12kb band on Eco R1 digests; Fig 3) without new rearranged bands was present in five of six T cell ALLs in this series, a pattern also noted in 11 of 15 T cell leukemias reported by Rabbitts et al.⁴⁴ Unlike polyclonal proliferations, rearranged bands are present on Bam H1 and Hind III DNA digests indicating both the monoclonal nature of ALL and the frequent use of the second TCR beta chain constant gene locus.⁴⁴ Whether this rearrangement pattern is a nonrandom event in some T cell ALLs is uncertain. It was not seen in any of our T cell lymphomas, all of which had rearranged bands on Eco R1 digests (except case no. 84-6).

Assays for rearrangements of K_{Del} proved to be very reliable in identifying Ig light chain gene rearrangements. The kappa constant or joining regions are often partially or completely deleted, especially in lambda-producing B cells.³⁵ The deletion of these sequences may not be apparent when studying DNA in which the tumor cells comprise only a small proportion of the total tumor sample, as nontumor cells will contribute to the germline bands on autoradiographs creating a false-negative assay. Specific assays for rearrangement of lambda light chain genes require the use of additional restriction enzyme digestions, and the interpretation of these assays is often difficult due to the high frequency of structural polymorphisms in this locus.^{49,50} We found no Bam H1, Eco R1, or Hind III restriction enzyme structural polymorphisms in the DNA near K_{Del} in the 15 normal samples that were tested. Since one K_{Del} allele is rearranged for each nonfunctional and deleted C_K gene, it should be possible to predict the presence of nonfunctional or potentially functional kappa genes.³⁵ The correlation of Ig light chain expression with the pattern of gene rearrangements in our B cell tumors generally supports this concept (Table 2). Since the K_{Del} and J_K (or C_K) germline bands are on separate Bam H1 fragments (14 Kb and 12 Kb, respectively) one could theoretically perform a single step Southern blot hybridization using a mixture of labelled K_{Del} and J_K (or C_K) probes to assess the presence of light chain gene rearrangements.

Several other observations made during this study deserve comment. First, the reliable detection of Ig and TCR gene rearrangements by Southern blot assay required analysis of separate samples of tumor DNAs that were digested with different restriction endonucleases, an observation that has been made by others.¹³ Second, none of the tumors contained Ig light chain gene rearrangement in the absence of heavy chain gene rearrangements, although such cases have been reported by others.⁵¹ Finally, the DNAs from all four T cell ALLs that expressed CALLA had evidence of TCR beta

chain gene rearrangements, though only one demonstrated a rearrangement in the Ig heavy chain genes. This observation is identical to that of Ginsburg et al,⁵² and verifies that expression of CALLa is not limited to B lymphocytes or B cell malignancies.

This work shows Ig and TCR gene rearrangements to be highly sensitive though not entirely specific in identifying lymphoid neoplasms of T or B cell origin. These assays have the potential to establish the cell lineage of phenotypically undifferentiated lymphomas and leukemias, and to detect monoclonal B or T cell populations in immunopathologically nondiagnostic lymphoproliferative lesions. However, the role of gene rearrangement assays in the clinical management of patients can only be determined by prospective clinicopathologic studies.

NOTE ADDED IN PROOF

Since submission of this manuscript, case no. 85-18 (Table 2) has been studied with the C_K probe. This showed that both C_K alleles were deleted as expected for a lambda-producing B cell tumor. However, one of the alleles may have been deleted by an aberrant mechanism since one K_{Del} element retained the germline configuration.

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Evidence Favoring Lineage Fidelity in Acute Nonlymphocytic Leukemia: Absence of Immunoglobulin Gene Rearrangements in FAB Types M4 and M5

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The concept of lineage fidelity in acute leukemia has recently been challenged by the finding of rearrangements of the immunoglobulin heavy chain genes in a leukemic cell line and in a small number of sporadic cases of acute nonlymphocytic leukemia with a monocytic phenotype. We therefore screened leukemic blood or bone marrow samples of 33 adult patients with acute nonlymphocytic leukemia of FAB types M4 (23 patients) and M5 (10 patients); 28 were obtained at diagnosis and 5 at relapse. All cases were well characterized pathologically and histochemically. Cytogenetic analysis performed in each case demonstrated karyotypes that were representative of those generally

seen in these types of leukemia, with a clonal abnormality present in all except 9 of 32 patients who were successfully studied. DNA prepared from each sample was digested with the restriction enzyme BamH1 and analyzed by Southern blot hybridization to probes for the J_H region of the immunoglobulin heavy chain. All 33 cases had DNA retained in the germline configuration with no evidence of rearrangement. This finding supports the concept of lineage fidelity, and suggests that true interlineage infidelity, myeloid to lymphoid, is a rare occurrence in adult acute nonlymphocytic leukemia.

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THE concept of lineage fidelity in acute leukemia states that the leukemic clone is derived from a single phenotypically defined cell, that markers with specificity for normal differentiation pathways maintain that specificity in the leukemic clone, and that differentiation is arrested rather than being deranged.¹⁻³ Furthermore, it implies that leukemic cells do not cross lineages. This concept remained largely unchallenged until recently when several investigators independently reported coexpression of lymphoid and myeloid markers in cells from some leukemic cell lines and from fresh leukemic tissue.⁴⁻⁶ This occurrence of "lineage infidelity" suggests that the leukemic cell may arise from a less well-differentiated stem cell precursor, which is capable of producing differentiated cells with features of both lineages.³ The investigation of leukemic lineage has led to many insights into the understanding of both normal and abnormal hematopoietic cell differentiation.

The hallmark of the B lymphocyte is the capacity to produce immunoglobulin. The loci for human immunoglobulin heavy, kappa, and lambda chain genes, have been mapped to chromosomes 14, 2, and 22, respectively.⁷⁻¹⁰ The genes coding for each immunoglobulin class are represented by a number of exons separated by noncoding regions along the chromosomes. For heavy chain genes at least four distinct elements are known, termed V (variable), D (diversity), J (joining), and C (constant) regions.¹¹ The primary event leading to expression of the gene is a somatic recombination of the germline elements such that these regions are apposed.¹² A developmental hierarchy exists such that heavy chain gene rearrangement represents the first stage of B cell commitment.¹³

Until recently, immunoglobulin gene rearrangements were considered to be restricted to normal B lymphocytes and B cell neoplasms. Demonstration of heavy chain gene rearrangements in cases of common "non-T/non-B" acute lymphoblastic leukemia (ALL),¹³ and cases of chronic myelogenous leukemia in lymphoid blast crisis¹⁴ and hairy cell leukemia^{15,16} indicate that these malignancies are of B cell origin. However, rearrangements of the heavy chain gene have also been detected in human and mouse T cell lines as well as in the leukemic cells of patients with T cell malignancies.^{12,17-19}

Heavy chain immunoglobulin gene rearrangements have been reported in one human acute myelogenous leukemia cell

line (ML), one cell line with both lymphoid and monocytoid features and a t(4;11) chromosomal rearrangement, as well as in fresh leukemic cells from three patients characterized phenotypically as acute nonlymphocytic leukemia (ANLL).^{20-22,32} Most of these cases had a monocytic phenotype. The finding of an irreversible stage of B cell commitment in some non-B cell neoplasms is surprising. Moreover, it casts doubt on the concept of lineage fidelity in leukemic blasts, and suggests instead that, in some cases of leukemia, transformation occurred in a progenitor cell capable of both B cell and myeloid differentiation, or that abnormal differentiation may include some switching between cell lineages.

To investigate this phenomenon, we studied leukemic cells from 33 adult patients with active acute myelomonocytic leukemia (AMMoL) or acute monoblastic leukemia (AMoL; FAB types M4 and M5)²² for the presence of immunoglobulin heavy chain gene rearrangements. This patient population was well characterized phenotypically and cytogenetically. We report that the immunoglobulin genes retained germline configuration in all cases.

MATERIALS AND METHODS

For cytogenetic analysis, fresh leukemic cell specimens were obtained from peripheral blood buffy coat or bone marrow from patients at the University of Chicago (23 patients) and from other institutions (10 patients). These patients all had active ANLL that

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had been classified as FAB type M4 or M5 by morphology and by cytochemical reactions according to established criteria.²³

Cytogenetic analysis was performed on cells obtained at the time of diagnosis or at clinical relapse as previously described.^{24,25} Metaphase chromosomes were analyzed using trypsin-Giemsa or quinacrine fluorescence banding techniques. Chromosome abnormalities were described according to the International System for Human Cytogenetic Nomenclature (1978).²⁶ An abnormal clone was defined by the detection of at least two pseudo-diploid or hyperdiploid cells, or at least 3 hypodiploid cells with identical chromosomal abnormalities.

High molecular weight DNA extracted from leukemic cell specimens that had been stored at -70°C was digested with the restriction enzyme Bam HI. The products were size-fractionated by electrophoresis in 0.8% agarose gels, and the DNA fragments were then transferred to activated nylon membranes (Gene Screen Plus, New England Nuclear, Boston, Mass) by the technique of Southern.²⁷ Membranes were hybridized as described previously,²⁸ with nick-translated ³²P-radiolabeled probes cloned in pBR 322 plasmids. Probes used recognizing the joining region of the immunoglobulin heavy chain gene (J_H -region) have been previously described.¹⁶ After

extensive washing of filters, autoradiography was performed against a sheet of x-ray film using a dual intensifying screen for 1 to 2 weeks at -70°C .

RESULTS

A total of 80 adult patients with a diagnosis of M4 or M5 ANLL were karyotyped at the University of Chicago between 1981 and 1985. Thirty-three of these patients had adequate numbers of leukemic cells frozen for DNA analysis and these constitute the study population. The median age of this group was 50 years (range 18 to 85 years). Twenty-three patients were characterized as AMoL (FAB type M4) including one patient with ANLL following refractory anemia. This patient (no. 214—see Table 1) had received low-dose cytosine arabinoside prior to the diagnosis of ANLL. Ten patients were characterized as AMoL (FAB type M5) including one patient who developed ANLL following chemotherapy for lung carcinoma. Twenty-eight patients had newly diagnosed ANLL and 5 patients were

Table 1. Karyotypes of Study Patients With M4 ANLL

Patient No.	Source	Status	% ABN* Cells	Karyotype
128	PB	dx	58	46,XY,t(8;11)(p11;p15),del(9)(q21q34)(100%).
148	BM	dx	60	46,XY(6%)/46,XY,-2,+der(2)t(2;11)(p25;q13), inv(16)(p13q22)(94%).
150	PB	rel	80	46,XX(83%)/48,XX,+8,+21,i(7q)(17%).
166	PB	dx	48	46,XY(100%).
168	BM	dx	83	46,X,del(Y)(q12),inv(16)(p13q22)(100%).
179	BM	dx	73	47,XY,+9,t(16;16)(p13;q22)(11%)/ 48,XY,+9,+22,t(16;16)(86%)/ SCA:48,XY,-8,+9,+9,+22,t(16;16)(3%).
182	BM	dx	49	46,XX(39%)/46,XX,del(7)(q32q36), inv(16)(p13q22)(61%).
183	BM	dx	76	46,XY,(16%)/46,XY,del(7)(q21q31), inv(16)(p13q22)(84%).
194	PB	dx	58	46,XX(100%).
195	BM	dx	80	46,XX(92%)/46,XX,del(6)(q17q23)(8%).
205	BM	dx	66	46,XY(14%)/46,XY,inv(3)(q21q26)(50%)/ 45,XY,-7,inv(3)(q21q26)(36%).
212	PB	dx	79	46,XY(100%).
214	BM	dx†	67	46,XX(93%)/46,XX,t(9;?)p(2?3;?) (7%).
215	BM	dx	50	46,XY(91%)/46,XY,del(16)(q22)(9%).
231	BM	dx	85	46,XX(100%).
238	BM	dx	70	46,XX(43%)/46,XX,inv(16)(p13q22)(57%).
8003	BM	dx	70	46,XX(13%)/46,XX,-16,-21,+der(21) t(16;21)(p11;q22),+mar(G size)(87%).
R-15	PB	rel	73	46,XY,inv(16)(p13q22),r(17)(p13q25)(59%)/ 47,XY,+8,inv(16)(26%)/46,XY,-17,+8, inv(16)(15%).
R-28	PB	dx	75	47,XX,+22,t(16;16)(p13;q22)(95%)/SCA:46,XX, t(16;16)(p13;q22)(5%).
R-29	BM	dx	95	47,XY,+22,t(16;16)(p13;q22)(27%)/46,XY, t(16;16)(p13;q22)(73%).
R-31	BM	dx	90	46,XY(5%)/47,XY,+i(11q)(58%)/46,XY,-16, +dic(11;16)(q13;p11),t(17;20)(p12;q11)(32%)/ SCA:47,XY,same,+10(5%).
R-32	BM	dx	90	47,XY,+Y(100%).
R-33	BM	dx	83	46,XX(100%).

Patients 128 to 183 and R-15 were previously reported.^{24,25} R refers to patient specimens referred to the University of Chicago. PB, peripheral blood; BM, bone marrow. Status: dx, diagnosis of leukemia; rel, relapse.

*Percentage of leukemic cells identifiable by morphology.

†Leukemia following refractory anemia.

Table 2. Karyotypes of Study Patients With M5 ANLL

Patient No.	Source	Status	% ABN Cells	Karyotype
164	BM	rel	92	46,XX,t(9;11)(p22;q23)(100%)
185	PB	dx	86	46,XX(97%)/SCA:47,XX,+8(3%)
186	BM	rel	91	46,XX(100%)
188	BM	rel	89	46,XX(81%)/57,XX,+6,+8,+8,+8,+10,+11+11, +13,+14,+18,+18(13%)/SCA:51,-X, ?del(X)(q11),-2,-4,-21,+6,+8,+8, +11,+11,+13,+14,+18,+18(6%)
200	BM	dx	84	46,XY(86%)/46,XY,ins(7;?) (p15;?) (14%)
2077	PB	dx*	69	46,XX(27%)/46,X,t(X;10)(p11;q11)(70%)/ SCA:46,X,t(X;10),t(17;?) (q27;?) (3%)
R-34	BM	dx	80	47,XY,+11,del(20)(q11.2q13.1)(100%)
R-35	PB	dx	92	Inadequate sample
R-36	PB	dx	86	46,XY(100%)
R-37	BM	dx	99	47,XY,+8,t(9;11)(p22;q23)(100%)

See Table 1 for notes.

*Therapy-related leukemia.

studied at the time of relapse. Leukemic cells constituted 48% to 99% of nucleated cells in the specimens obtained, as assessed by morphology (peripheral blood in 11, bone marrow in 22 patients).

The karyotypic abnormalities identified in these patients were consistent with those generally seen in these types of leukemia (see Tables 1 and 2).²⁹ Specifically, the inv(16) or t(16;16) that has been associated with AMMoL^{25,29,30} was observed in 9 patients, and the t(9;11), a rearrangement commonly seen in AMoL, was noted in 2 patients. Chromosomal anomalies seen in other types of ANLL, such as the t(8;21) and t(15;17) in M2 and M3, respectively, were not seen in this group. One patient (no. 188) who had a hyperdiploid clone with 57 chromosomes had an extra chromosome 14; otherwise no abnormalities involving chromosome 14 (site of the heavy chain gene) were found. This observation is not surprising since nonrandom abnormalities of chromosome 14 have not been reported in ANLL. A gain of chromosome 22 was observed in 3 patients. In each case this abnormality was associated with an inv(16) or a t(16;16), a phenomenon that has been reported previously.²⁹ A single patient (no. 148) had a rearrangement involving chromosome 2; however, this rearrangement involved a breakpoint in band p25, a site that is distal to the immunoglobulin light chain locus at p11-p13. Only 9 patients failed to show a clonal abnormality.

All 33 cases were analyzed for immunoglobulin gene rearrangements. In each case DNA was retained in the germline configuration (single 18 kilobase fragment) with no evidence of rearrangements (see Fig 1).

DISCUSSION

In our study, all of the adult patients with the M4 or M5 subtype of ANLL clearly retained the immunoglobulin heavy chain gene in its germline configuration. We decided to investigate the monocytic and myelomonocytic subgroups, since immunoglobulin gene rearrangements in ANLL seem to be more common in these subgroups. All our patients were well characterized as M4 and M5 and no patients had ambiguous markers or lymphoid phenotypes. These findings

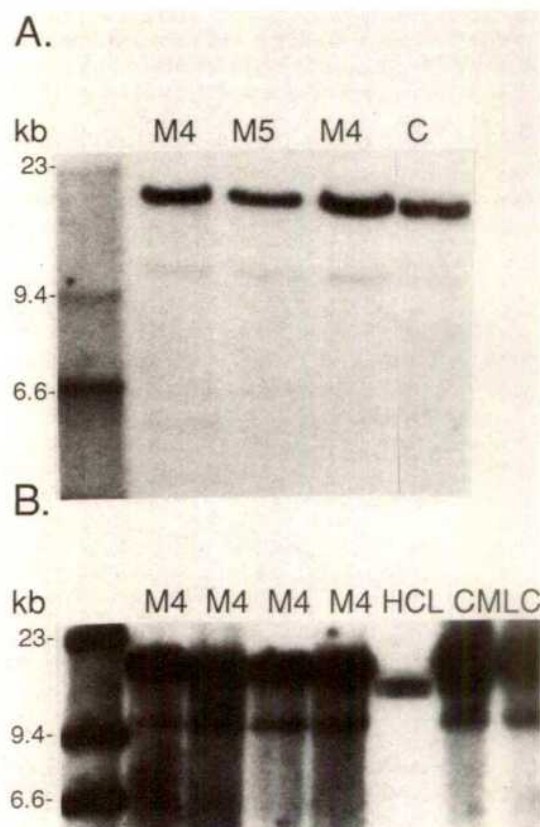


Fig 1. (A) Southern blot analysis of BamHI digests from leukemic cells of 7 patients with acute myelomonocytic (M4) or acute monoblastic (M5) leukemia, and controls (C, human placental DNA, CML, peripheral leukocyte DNA from a patient with chronic myeloid leukemia, HCL, peripheral leukocyte DNA from a patient with hairy cell leukemia). Blots were hybridized with a J_H -specific probe. All patients with ANLL retained the germline 18kb fragment. (B) Rearranged J_H genes from a patient with hairy cell leukemia are shown as a positive control. Lane 1: fragment size markers at 23, 9.4, and 6.6 kilobases. (The faint band seen at 10.5 kb in all but one lane is artifactual, shown by rehybridization to be contaminating plasmid DNA—data not shown.)

are in contrast to two recent series; Rovigatti et al²² demonstrated a $C\mu$ segment rearrangement in 2 of 14 samples examined from children with ANLL, and Ha et al²⁰ found a rearrangement in one adolescent patient classified as FAB type M1, but only germline configuration of immunoglobulin genes in 8 other children and 10 adults with ANLL.

Of six myeloid cell lines studied to date, only one (ML) has shown a $C\mu$ rearrangement; HL-60, K562, U937, THP1, and KG-1 show no such rearrangement.²⁰⁻²² The cell line established from a 32-year-old leukemic patient with a t(4;11) translocation also had a $C\mu$ rearrangement.³² This information is difficult to interpret because this cell line simultaneously displays lymphoid and monocytic features. Patients with this karyotype may be diagnosed as acute lymphoblastic, acute myelomonocytic, or biphenotypic leukemia.^{31,32}

In a variety of hematopoietic malignancies that have nonspecific surface markers, the presence of rearrangement

of immunoglobulin genes from the germline configuration is considered a marker of clonal B cell differentiation.¹³⁻¹⁶ However the observations of such rearrangements in a few cases of myeloid leukemia have led to the suggestion that the initial event in immunoglobulin gene organization occurs during an early stage of hematopoietic cell development, at a time when the cells are not restricted to B lineage development, but may differentiate along more than one pathway.²⁰ This concept is supported by the demonstration of heavy chain rearrangements in an occasional T cell neoplasm.^{13,17-19}

All of these exceptions raise questions regarding the concept of lineage fidelity in acute leukemia. In contrast, our findings support the concept of lineage fidelity, and suggest instead that true interlineage infidelity, myeloid to lymphoid, is either an extremely uncommon occurrence in adult ANLL, or that its appearance is confined to childhood ANLL.

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Treatment of Preleukemic Syndromes With Marrow Transplantation

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Thirty patients with advanced preleukemic syndromes were treated with marrow transplantation. Most cases were diagnosed by the presence of peripheral pancytopenia and a diagnostic marrow examination but in 6 of the 30 patients pretransplant chromosome studies were instrumental in establishing the diagnosis. Three patients prepared for transplantation with cyclophosphamide alone recurred with their disease within 6 months of transplantation. The other 27 patients were treated with cyclophosphamide and total body irradiation. Twenty of these 27 patients had preleukemia not associated with prior therapy or severe marrow fibrosis. Thirteen of these 20 are alive and well 9 to 56 months from transplant and 7 died, 4 of interstitial pneumonia, 2 of candida septicemia, and 1 of

disseminated zoster. There have been no disease recurrences in this group. The remaining preleukemic patients, which include 3 patients transplanted for preleukemia secondary to prior therapy and 4 patients transplanted for preleukemia associated with severe marrow fibrosis, have all died. Major problems in these patients included disease recurrence (2 cases) and, in those with severe marrow fibrosis, graft failure (2 cases). These results suggest that for patients with life-threatening pancytopenia due to spontaneous preleukemia without severe marrow fibrosis, marrow transplantation can prolong disease-free survival and may result in cure of the disease.

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PRELEUKEMIA describes a group of bone marrow disorders characterized by abnormal maturation of hematopoietic cells and the development of peripheral blood cytopenia.¹⁻⁶ Although the clinical course of preleukemia is variable, as many as 75% of patients will develop acute leukemia within 24 months of diagnosis and many others die of complications related to thrombocytopenia or leukopenia before overt leukemia develops.¹⁻⁶ Unfortunately, few therapeutic options exist for the patient with preleukemia. Androgens, pyridoxine, prednisone, and standard cytotoxic therapy are rarely of benefit.⁷⁻⁹ More recently the use of low-dose cytarabine has been shown to result in meaningful clinical responses in some patients.¹⁰⁻¹² However, these responses have almost always been incomplete and temporary with no suggestion of curative potential.

Marrow transplantation is an effective therapy for a number of malignant and nonmalignant marrow diseases. We therefore initiated a clinical trial of the use of marrow transplantation in patients with preleukemia and life-threatening cytopenia. Results in ten cases have been reported.¹³ Thirty patients have now been treated. In this report the toxicities and therapeutic results of marrow transplantation for preleukemia are described.

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PATIENTS AND METHODS

Thirty consecutive patients with preleukemia were treated between May 1977 and December 1985. In this study we included as "preleukemic" patients with pancytopenia and the presence of myelodysplasia on marrow examination, patients with aplastic marrows and a clonal cytogenetic abnormality of a type associated with hematologic malignancies, or patients with otherwise unexplained myelofibrosis. Table 1 shows the clinical characteristics of the patients. Ages ranged from 4 to 54 years (median 24 years) with 17 males and 13 females. In 3 patients (UPN 2798, 2799, and 2813) preleukemia was apparently therapy related, in 3 cases (UPN 2173, 2855, and 1763) preleukemia occurred in patients with Fanconi's anemia, and one patient (UPN 1694) had a family history of preleukemia. No cause was identified in the other 23 cases. The median duration of disease from diagnosis to transplantation was 12 months and ranged from 2 to 180 months. All patients received transfusion support prior to referral and 19 had received prior therapy, the most common forms of therapy being steroids (16 patients), androgens (6 patients), and low-dose cytarabine (4 patients). At the time of referral for transplantation all patients required red cell transfusion support, 26 required platelet transfusions, and 25 had a granulocyte count of less than 1,000/cu mm. Peripheral blood and marrow samples were reviewed and classified, when possible, according to the proposals of the French-American-British (FAB) cooperative group.¹⁴ Degree of marrow fibrosis was graded according to the criteria of Bauermeister.¹⁵ Twenty-one patients had disease that could be classified as myelodysplastic according to the proposed FAB schema including 9 patients with refractory anemia and 12 patients with refractory anemia with excess blasts. Six patients had aplastic marrows but a clonal cytogenetic abnormality. Two patients had unexplained myelofibrosis. A final patient had a normal appearing marrow but severe pancytopenia and trisomy 8.

Marrow samples were processed for chromosome analysis after 1.5 and 24 hours in culture without added mitogens. In 3 patients (UPN 2208, 2254, and 2813) marrow either could not be aspirated or no metaphases were present for analysis. Chromosome banding was done using quinacrine mustard for Q bands.

Details of the transplant procedure are presented in Table 2. Histocompatibility between donor and recipient was determined by HLA typing and mixed leukocyte culture tests. In 24 cases the donor was an HLA-identical sibling, in 1 case an HLA phenotypically matched father, and in 5 cases a son or sibling mismatched for 1 (2 cases) or 2 (3 cases) antigens.

The technique of marrow transplantation has been described.¹⁶

Table 1. Pretransplant Clinical Characteristics of 30 Patients With Preleukemic Syndromes

UPN	Age	Sex	Disease Duration (mo)	Previous Treatment	Hgb	WBC	PMN	Plt	Cell*	Marrow		Cytogenetics	CMV Titer†
										Fibrosis	Morph		
691	9	F	39	A,S	9.4	3,600	468	2,000	1+	0	AA	45,XX,-7	—
1042	30	M	2	—	5.0	3,125	656	9,700	4+	0	RAEB	45,XY,-7	—
1442	34	F	3	S	7.3	1,600	120	13,000	1+	0	AA	46,XX,ins(3;3)(q26;q21q26)	+
1558	13	M	26	S,P	5.5	1,400	84	20,800	0+	0	RA	45,XY,-7	+
1636	54	M	3	S	5.2	2,200	320	3,000	1+	0	RAEB	46,XY,-5,-6,-7,-17,-21,+mar(1-4)	—
1694	4	F	46	S	8.4	3,425	206	6,000	0+	0	AA	45,XX,-7	+
1763	11	F	50	—	8.0	2,400	744	5,100	2+	0	RA	48,XX,+8,+20	—
1779	12	F	2	A,S	8.3	1,600	80	1,700	3+	0	RAEB	46,XX,del(7)(q32q36)	—
1815	14	M	27	S,ATG	5.0	3,000	690	2,800	0+	0	AA	45,XY,-7	—
1996	11	M	6	S	10.0	2,700	490	11,000	3+	0	RA	46,XY	—
2016	30	M	6	S,P	9.8	800	70	10,600	3+	4+	RAEB	46,XY	+
2088	18	M	48	—	9.1	2,200	374	39,000	4+	0	RA	45,XY,-7,-17,+i(17q)	+
2173	19	F	36	—	11.0	2,000	500	41,000	3+	0	RAEB	45,X,-X,del(5)(q13q33)	+
2208	25	F	10	A,S	8.0	2,550	1,860	9,500	1+	4+	MF	NA	—
2216	32	M	52	—	10.6	1,600	480	8,900	0+	0	RA	46,XY	+
2254	36	M	12	—	10.0	8,600	7,740	732,000	2+	4+	MF	NA	—
2482	24	M	2	S	9.0	2,300	230	9,100	0+	0	AA	47,XY,+8	—
2586	27	F	13	Endo	8.0	1,400	504	180,000	2+	0	RAEB	46,XX,ins(3;3)(q26;q21q26),del(22)(q12)	—
2602	8	F	9	LDAC	7.5	1,600	60	8,000	1+	3+	RAEB	45,XX,-7	+
2633	34	F	180	A,S,ATG,LDAC	7.0	2,000	462	9,000	2+	0	RA	46,XX	+
2635	47	F	7	A,S,LDAC	7.8	2,000	180	15,000	4+	0	RA	49,XX,+mar(1-3)	+
2798	41	F	120	—†	8.1	2,000	460	400	1+	0	RA	46,XX	+
2799	22	M	10	—†	11.2	8,400	2,100	65,000	4+	0	RAEB	46,XY,-5,-7,-20,+22,+mar(1-2)	—
2813	36	M	4	—†	9.3	1,800	320	15,000	4+	1+	RAEB	NA	—
2814	18	M	2	—	9.7	3,900	2,700	5,200	4+	1+	RAEB	46,XY	+
2836	20	F	7	HDAC	9.0	1,200	450	23,000	3+	0	RAEB	47,XX,+8	+
2853	24	M	156	A,S	10.0	1,900	60	10,000	1+	0	AA	48,XY,-7,+8,+21,+22	—
2855	22	M	154	—	8.8	1,400	378	1,100	3+	0	Normal	47,XX,+8	—
2904	45	M	60	S,LDAC,CRA	9.7	6,500	2,200	57,000	2+	2+	RA	46,XY	+
3060	32	M	52	S,CSP	10.3	2,000	720	21,000	3+	0	RAEB	45,XY,-7	—

Abbreviations: UPN, unique patient number; A, androgens; S, steroids; P, pyridoxine; ATG, antithymocyte globulin; Endo, endotoxin; LDAC, low-dose cytarabine; CRA, cis retinoic acid; HDAC, high-dose cytarabine; CSP, cyclosporine; AA, aplastic anemia; RAEB, refractory anemia with excess blasts; RA, refractory anemia; MF, myelofibrosis; NA, marrow not aspirable.

*0, acellular; 1, markedly hypocellular; 2, mildly hypocellular; 3, monocellular; 4, hypercellular.

†Patients treated for a prior malignancy; UPN 2798 with busulfan; UPN 2799 and 2813 with nitrogen mustard, vincristine, prednisone, procarbazine, and irradiation.

‡Presence (+) or absence (—) pretransplant of antibody to cytomegalovirus.

The preparative regimens used prior to transplantation were those we have used for treatment of aplastic anemia (UPN 691, 1042, and 1442) or acute leukemia (the remaining 27 patients).^{17,18} All patients received posttransplant immunosuppression with either methotrexate (4 cases), cyclosporine (1 case), or a combination of the two (25 cases) in an effort to diminish or prevent graft-v-host disease (GVHD).^{19,20}

Patients were also entered on other protocols involving laminar air flow isolation and prophylactic systemic antibiotics. All risks of the treatment protocol were fully explained to patients, donors, and relatives. Informed consent was obtained using forms approved by the Investigational Review Board of the Fred Hutchinson Cancer Research Center. Data were analyzed as of July 1, 1986.

RESULTS

The results are summarized in Table 2. Three patients (UPN 691, 1042, and 1442) were prepared for transplantation with cyclophosphamide alone and, as previously reported, in all three cases there was evidence of either persistence or recurrence of the abnormal cell clone within 6 months of transplantation. Despite further chemotherapy (1 patient) or second marrow transplants (2 patients), all three patients died within 9 months of initial transplant.

All other patients were prepared for transplantation with a regimen consisting of cyclophosphamide and total body

irradiation. Three patients (UPN 2798, 2799, and 2813) were transplanted for therapy-related preleukemia, 2 after chemoradiotherapy for Hodgkin's disease, and 1 after prolonged busulfan therapy for polycythemia vera. All three died within 3 months of transplant, one of persistent disease, one of septic shock, and one of venoocclusive disease of the liver and candida septicemia. Four patients were transplanted for preleukemic syndromes characterized by extensive marrow fibrosis. None of these 4 survive with one patient dying of recurrent disease, 2 with graft failure associated with either bleeding or infection, and one of idiopathic interstitial pneumonia. Twenty patients were transplanted for preleukemia which was neither therapy related nor associated with extensive marrow fibrosis. Thirteen of these 20 patients are alive without either clinical or cytogenetic evidence of disease from 9 to 56 months after transplant. The seven patients who failed therapy included 4 cases of interstitial pneumonia (3 idiopathic and one associated with cytomegalovirus; CMV), 2 cases of candida septicemia, and one case of disseminated herpes zoster infection. None of the 20 patients suffered a recurrence of their disease.

In addition to the apparently important influences of the preparative regimen, etiology of disease, and presence of marrow fibrosis, patient age seemed to influence survival

Table 2. Marrow Transplantation in 30 Patients With Preleukemic Syndromes

UPN	Preparative Regimens	GVHD Prophylaxis	Donor	Results
691	CY	MTX	HLA = sib	Relapse d194, death d261
1042	CY	MTX	HLA = sib	Relapse d170, death d286
1442	CY	CSP	HLA = sib	Resistant disease, death d210
1558	CY + 14 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d1647
1636	CY + 10 Gy TBI	CSP + MTX	HLA AB ≠ son	IP, death d128
1694	CY + 12 Gy TBI	MTX	HLA = father	Alive and well, d1496
1763	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d1412
1779	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d1387
1815	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d638
1996	CY + 12 Gy TBI	MTX	HLA = sib	Alive and well, d1152
2016	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Relapsed, death d569
2088	CY + 12 Gy TBI	CSP + MTX	HLA D ≠ sib	Alive and well, d1056
2173	CY + 12 Gy TBI	CSP + MTX	HLA = sib	IP, death d12
2208	CY + 12 Gy TBI	CSP + MTX	HLA = sib	IP, graft failure, death d71
2216	CY + 12 Gy TBI	CSP + MTX	HLA D ≠ sib	IP-CMV, death d50
2254	CY + 12 Gy TBI	CSP + MTX	HLA = sib	IP-CMV, death d57
2482	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d719
2586	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d636
2602	CY + 12 Gy TBI	CSP + MTX	HLA BD ≠ sib	Graft failure, bleed, death d113
2633	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Candida sepsis, death d74
2635	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d580
2798	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Septic shock, death d63
2799	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Resistant disease, death d76
2813	CY + 12 Gy TBI	CSP + MTX	HLA = sib	VOD, Candida sepsis, death d14
2814	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d428
2836	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d410
2853	CY + 12 Gy TBI	CSP + MTX	HLA BD ≠ sib	GVHD, death d66
2855	CY + 12 Gy TBI	CSP + MTX	HLA = sib	IP, death d39
2904	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Disseminated zoster, death d21
3060	CY + 12 Gy TBI	CSP + MTX	HLA = sib	Alive and well, d251

Abbreviations: UPN, unique patient number; CY, cyclophosphamide; TBI, total body irradiation; GVHD, graft versus host disease; MTX, methotrexate; CSP, cyclosporine; IP, interstitial pneumonia; CMV, cytomegalovirus; VOD, venoocclusive disease of the liver.

($P = .047$, log rank test). We could detect no influence of disease duration, marrow cellularity, marrow morphology (except for extensive fibrosis), or cytogenetic findings on treatment outcome. Of 6 patients receiving grafts from other than genotypically matched donors, two remain alive. Three patients (UPN 2173, 2855, and 1763) had Fanconi's anemia preceding the development of preleukemia. One of these (UPN 1763) tolerated the transplant procedure well while the other 2 developed early severe toxicity and died of interstitial pneumonia.

The health of the 13 disease-free survivors is excellent. All have normal hematologic function and 12 of the 13 are off all medications with Karnofsky scores of 90% to 100%. One patient has developed bronchial asthma posttransplant without other evidence of chronic graft-v-host disease. He requires no immunosuppressive therapy.

DISCUSSION

Despite recent advances in the management of patients with preleukemic syndromes, in particular the use of low-dose cytarabine, patients with preleukemia are generally considered to be incurable and most of these patients die with bleeding, infection, or acute leukemia. The success of high-dose chemotherapy or chemoradiotherapy followed by marrow transplantation as treatment for various marrow disorders

prompted us to study this approach in patients with preleukemic syndromes.

The first 3 patients were given cyclophosphamide alone as a preparative regimen, similar to the way in which patients with aplastic anemia are prepared for transplantation. Although all three demonstrated evidence of engraftment of donor marrow, in all three the malignant clone recurred suggesting that cyclophosphamide alone, or combined with any allogeneic reactivity of the engrafted marrow, is inadequate to eradicate the abnormal cell population. Accordingly, subsequent patients were treated with cyclophosphamide and TBI.

Results obtained with a combination of cyclophosphamide and TBI in patients with preleukemia unassociated with previous therapy or severe marrow fibrosis were encouraging. Thirteen of 20 patients are alive in complete remission up to 56 months from therapy. Importantly, there has not been a single episode of disease recurrence in this group. Although continued follow-up is necessary, this result suggests that the preparative regimen used, cyclophosphamide 60 mg/kg for two days followed by 200 cGy/day of TBI for six days, is adequate to eradicate the abnormal cell population. The major reason for treatment failure was interstitial pneumonia in 4 patients, in one case associated with CMV and in 3 cases idiopathic. These 4 patients included 2 with a history of Fanconi's anemia and 2 transplanted from mis-

matched donors. Since recurrent disease has not been a major problem and since irradiation presumably contributed to the observed toxicities, the use of lower doses of TBI or lung shielding might be considered in patients undergoing transplantation for preleukemia.

Results in the small group of patients with preleukemia secondary to previous therapy were less encouraging. One patient never cleared his abnormal cell population and the other 2 died of transplant-related complications. This unfavorable experience is apparently not universal. Gyger et al reported a 22-year old man who developed preleukemia following chemoradiotherapy for Hodgkin's disease who received an HLA-identical marrow graft from his sister following treatment with busulfan and cyclophosphamide. He was reported to be alive in remission 311 days posttransplant.²¹

Results in the 4 patients transplanted for preleukemia associated with extensive marrow fibrosis were discouraging. None of these patients is alive, one having relapsed and 3 having died of transplanted-related complications. Further, in 3 of these 4 patients normal peripheral blood counts were never achieved despite evidence for lymphoid engraftment and in 2 of these 3, pancytopenia was the cause of death. There are several case reports that demonstrate that engraftment can be achieved in some patients with severe marrow fibrosis.²²⁻²⁶ But, as case reports, these studies did not compare the outcome of transplantation in patients with and

without marrow fibrosis. The question of the effect of severe marrow fibrosis on engraftment prompted us to review our wider experience with transplantation in patients with marrow fibrosis of any etiology. As recently reported by Rajantie et al, patients transplanted for preleukemia, chronic myelogenous leukemia, or acute leukemia with coexistent extensive marrow fibrosis frequently have difficulty with engraftment as evidenced by a graft failure rate of 33% and slower myeloid and platelet engraftment in those who do engraft when compared to age- and disease-matched control patients with little or no fibrosis.²⁷

The experience reported here emphasizes the importance of marrow cytogenetic results in deciding on treatment of patients with pancytopenia. Six of the 30 patients described here were thought on initial morphologic examination of the marrow to have aplastic anemia. In each case marrow cytogenetics showed a clonal chromosomal abnormality establishing the diagnosis of preleukemia, including 3 cases of monosomy 7, one case of trisomy 8, one case with 46,XX,ins(3;3)(q26;q21q26), and one case with 48XY,-7,+8,+21,+22.

Patients with preleukemia and life-threatening pancytopenia, in general, do poorly. The results reported here, particularly in those patients with de novo preleukemia without severe marrow fibrosis, suggest that if an appropriate donor is available, marrow transplantation should be strongly considered.

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Cytogenetic and Histologic Correlations in Malignant Lymphoma

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Although a number of studies have indicated correlations between histologic subtypes of tumors and certain nonrandom chromosome changes, cytogenetic studies of lymphoma are in an early stage compared to those of leukemia. No comprehensive analysis of available data has so far been attempted in the literature either. Here we present an analysis of chromosome changes and their correlation with subtypes of lymphoma studied by conventional histology and cell surface markers, as observed in two sets of data: (1) a group of 65 karyotypically abnormal tumors sequentially ascertained and studied by us during the period January 1, 1984 to April 30, 1985, and (2) a larger data set derived by combining our data with those from two published series from the University of Minnesota that are comparable to our data. These combined data, which

comprise the largest data set on the cytogenetics of lymphomas assembled so far, enabled a comprehensive analysis of correlation between chromosome change and tumor histology and the patterns of chromosome instability in these tumors. We found several significant associations, some previously described and others now recognized, between nonrandom chromosome gains, breaks, translocations, and deletions and histologic subtypes of tumors that characterize lymphomas. The data indicate that finding of chromosome breaks at certain sites (eg, 8q24, 14q32, 18q21) is of diagnostic value in dealing with cases of unusual lymphoma. Furthermore, nonrandom chromosome breakage exhibited three distinct patterns that reflected three levels of etiologically relevant genetic change.

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HISTOLOGIC and immunologic studies of malignant lymphoma have led to a number of systems of classifications that have attempted to define subgroups based on biological relatedness or clinical significance.¹⁻⁴ Recent molecular studies of Burkitt's lymphoma and chronic myelogenous leukemia have shown that chromosome translocations play an important role in the development of neoplasia by bringing about deregulation of the genes involved in rearrangements.^{5,6} Cytogenetic studies of leukemia further have established that specific chromosome abnormalities are diagnostic and prognostic indicators.^{7,8} The clinical significance of cytogenetic changes in lymphomas is less clear⁹⁻¹² compared to leukemias where chromosome analysis is now performed as part of the clinical evaluation of patients. A number of recent studies however have demonstrated that recurring translocations t(14;18)(q32;q21) and t(8;14)(q24;q32) and the recurring deletion del(6q) are associated with certain histologic subtypes of lymphomas.¹³⁻¹⁶

We have been conducting a prospective study of correlations between chromosome changes, histology, and immunological markers in tumors from all patients with a presumptive diagnosis of lymphoma seen by the Hematology/Lymphoma Service of Memorial Sloan-Kettering Cancer Center (MSKCC) since January 1, 1984. Although, as mentioned above, previous studies have addressed translocations and deletions associated with non-Hodgkin's lymphoma (NHL), a comprehensive review of cytogenetic abnormalities associated with lymphoma has been lacking. By utilizing a combination of our data with that reported by two groups from the University of Minnesota,¹³⁻¹⁶ each of which included more than 50 karyotypically abnormal tumors classified by the Working Formulation (WF; Table 1), we have been able to assemble the largest data set examined so far and undertake such a review. Our analysis confirms the previously reported associations as well as establishes new correlations. In addition, we have been able to define classes of chromosome instability in lymphoma that indicate specific mechanisms of gene perturbation that are of possible significance in lymphoma development.

MATERIALS AND METHODS

Patient population and tumor material. During the study period we have analyzed 110 histologically confirmed lymphomas removed from 110 patients whose ages ranged from 7 to 80 years (mean 50 years). Of these, 58 were males and 52 were females. In 79 patients the tumor was studied at primary diagnosis, while in 25 it was studied during relapse following previous chemotherapy or radiation therapy. Previous treatment history was unknown in the remaining 6 patients. In each of three patients, two separate specimens were received yielding a total sample of 113 specimens. Involved tissues included lymph nodes in 59, lymph node and pleural fluid in one, spleen in 16, nonlymphoid tissues (fibrous tissue, muscle, lung, etc) in 22, bone marrow in 13, and ascites alone in one patient. A piece of each tumor received in Pathology was submitted for independent studies of histology, immunological markers, and cytogenetics; in addition, a small piece was frozen immediately and stored in liquid nitrogen for molecular studies. No criteria other than availability of tissue were applied in selecting patients for study. Because of this, 24 benign lymphadenopathies and 13 Hodgkin's disease lymph nodes also were examined during this period. The data pertaining to these specimens are not discussed here. In all cases, the tissue was processed within one hour of removal from the patient. All tissues used in this study were submitted to the laboratory for diagnostic evaluation. As such, they were removed from the patients

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Table 1. Numbers of Tumors With Clonal Karyotypic Abnormalities in Different Histologic Subtypes of Malignant Lymphoma in CD

Reference	Low Grade			Intermediate Grade				High Grade			Unclassified	Total
	SmLy	SCC-F	Mx-F	LC-F	SCC-D	Mx-D	LC-D	SNCC	Imb	Lyb		
13	11	14	2	3	1	2	3	3	3	—	—	42
14	8	11	3	1	0	7	7	0	2	1	1	41
15	16	21	4	9	1	10	18	6	3	2	2	92
16	3	6	3	4	—	—	7	—	0	1	—	24
Present report	3	10	4	3	4	1	19	3	3	1	14	65
Total	41	62	16	20	6	20	54	12	11	5	17	264

Abbreviations: SmLy, small lymphocytic; SCC, predominantly small cleaved cell; Mx, mixed small and large cell; LC, predominantly large cell; SNCC, small noncleaved cell; Imb, large cell immunoblastic; Lyb, lymphoblastic; F, follicular; D, diffuse.

after obtaining informed consent for evaluation at the time of admission to Memorial Hospital.

Histology and immunological markers. Histologic preparations were made from solid tumor tissue usually fixed in B3 and stained with hematoxylin and eosin using standard methods. Lymphomas were classified according to the WF, Rappaport, Lukes-Collins, and Kiel systems.¹⁻⁴ Bone marrow and effusion specimens were examined only to ascertain the presence of lymphoma. Bone marrow biopsies were embedded in Epon plastic and stained with Giemsa. The WF classification was followed in describing tumors in this paper in order to facilitate comparison of our data with those from other published series.¹³⁻¹⁶ In the text we have referred to the different lymphoma subsets by their abbreviations as indicated in Table 1. Cell surface phenotypes were studied in cell suspensions of solid tumors (lymph node, spleen, soft tissues) while surface as well as cytoplasmic phenotypes were studied on bone marrow tumors. The methods used for these studies included study of conventional markers for lymphoid cell subpopulations (IgG-FC, C₃ receptors, heavy and light chain immunoglobulins, and rosetting with sheep and mouse red cells) as well as typing with a panel of monoclonal antibodies (OK-T1, OK-T3, OK-T4, OK-T6, OK-T8, OK-T11, J-5, OK-Ia, B1, B2, and OK-M1).

Cytogenetics. The tumor specimen was minced into a single-cell suspension immediately upon receipt in the laboratory under sterile conditions in prewarmed RPMI 1640 medium. The cells were pelleted by centrifugation at 400 rpm for ten minutes, and resuspended in the same medium supplemented with 20% heat-inactivated fetal calf serum, 1% penicillin-streptomycin, and 1% L-glutamine. Cell viability was determined by trypan blue exclusion, and the cell concentration was adjusted to approximately 10⁶ living cells/mL. A direct harvest was made from one 10 mL cell suspension after exposure to 0.05 µg/mL colcemid for ½ hour at 37 °C. A second 10 mL cell suspension was incubated at 37 °C overnight with 0.025 µg/mL colcemid and harvested, and a third 10 mL cell suspension was incubated overnight at 37 °C, then exposed to 0.05 µg/mL colcemid for one hour and harvested. Cell harvesting was done following procedures used for chromosome preparations from blood lymphocytes. Quinacrine banding was employed throughout in karyotyping. For each patient with a successful culture, 15 to 30 cells were analyzed under the microscope for changes in chromosome number and structure. This was followed by analysis of photographic prints of at least five cells in the case of tumors with a single abnormal clone and ten cells in the case of tumors with more than one abnormal clone; three formal karyotypes were prepared for each clone. The karyotypes were described according to ISCN.¹⁷ (The detailed tabulation of tumor histology, cell surface markers, and karyotypes of all tumors studied by us is not published here but is available upon request from R.S.K. Chaganti).

Statistical analysis of data. We enumerated all identified chromosome abnormalities (gains and losses of individual chromosomes, translocations and deletions, and breakpoints of structural altera-

tions) from all tumor karyotypes from our data as well as from the combined data (CD). We tested for the nonrandomness of numerical and structural chromosome changes using the computer program for statistical analysis of chromosome changes written by Brodeur et al.¹⁸ This program tests the significance of observed deviation from the expected on two assumptions: (1) in the generation of numerical alterations all chromosomes have the same probability of undergoing nondisjunction, and (2) in the generation of structural alterations the frequency of chromosome breaks occurs proportional to the length of the chromosome arm. Conventional 2 × 2 chi-square analysis was employed to compare the frequency of occurrence of chromosome abnormalities between different histologic subtypes.¹⁹ The latter method has been used by Levine et al¹⁶ in their analysis of chromosome changes in lymphomas.

RESULTS

Histologic classification of part of the same tissue specimen that was used for cytogenetic and immunological marker studies was accomplished in 98 cases that presented as solid tumors. Cytogenetic analysis was performed on bone marrow and ascites specimens in only 14 cases. These were considered unclassified lymphoma for the purpose of this study. Cell surface markers established B cell type in 74 (86%) tumors, T cell type in four (4.7%), and null cell type in six (7%); the results were inconclusive in two (2.3%) cases. Marker studies were not performed in the remaining 27 tumors. IgM was expressed by 45 (60.1%) and IgG by 16 (21.6%) B cell tumors. More than one heavy chain was expressed in seven tumors. The kappa and lambda light chain markers were expressed nearly equally (36 and 30 tumors, respectively). The four T cell tumors were of postthymic (or peripheral T cell type) and histologically identified as large cell or immunoblastic types. No case of lymphoblastic T cell lymphomas were encountered in this study.

In our series, 77% of the specimens yielded sufficient numbers of analyzable metaphases, a cytogenetic success rate that is comparable to the 68% and 65% reported by Bloomfield et al¹⁵ and Kaneko et al,¹² respectively, although Yunis et al^{13,14} reported a 93% to 95% success rate in their series. Among the 87 successfully karyotyped specimens, five follicular and 16 diffuse tumors (24% of total successful cases) showed only normal karyotypes. Therefore these were considered as the karyotypes of the proliferating reactive cells in the malignant tumor and were not included in the statistical analysis of the data. The remaining 66 specimens (65 tumors) showed clonal karyotypic abnormalities. A mixture of normal and clonally abnormal cells was present in five follicular and 12 diffuse tumors. More than one abnor-

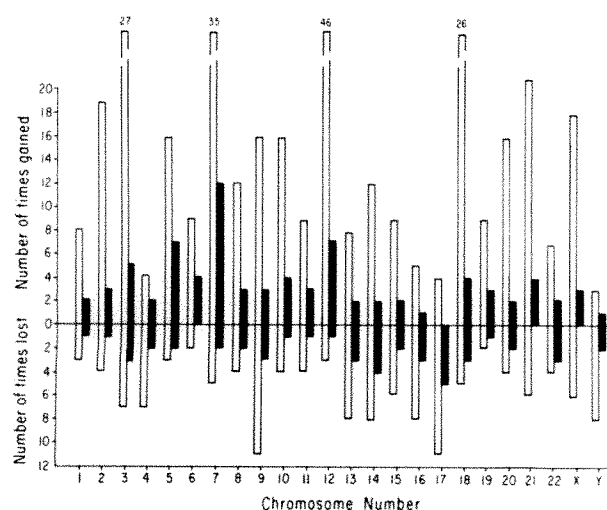


Fig 1. Losses and gains of chromosomes in malignant lymphoma. While none of the losses was statistically significant, gains of chromosomes 5, 7, and 12 in the MSKCC data and gains of chromosomes 3, 7, and 12 in the CD were statistically significant. □, Combined data; ■, MSKCC data.

mal clone was seen in two follicular and four diffuse tumors. Tumors from treated and untreated patients did not show significant differences in chromosome abnormalities. Therefore, the data are presented together. For the analysis of correlation between histology and cytogenetics, the 51 tumors for which both sets of data were available only were considered while chromosome instability was analyzed from the entire dataset.

Numerical changes. Although loss of different chromosomes was noted in 14 tumors in our data, a consistent correlation with histology was not seen. With regard to gains, 29 tumors showed trisomy of 21 different autosomes (Fig 1). Trisomy of chromosomes 7, 5, 11, and 12 were most frequent, and showed strong histologic correlations. Of 19 cases of LC-D, six showed trisomy 7 and five showed trisomy 12. Of the nine cases of diffuse large cleaved cell lymphoma (LCC-D), a subgroup of LC-D, three showed trisomy 11, and of the 10 cases of diffuse large noncleaved cell lymphoma (LNCC-D), another subgroup of LC-D, three showed trisomy of chromosome 5.

Among the tumors in the CD, significant deviation from

expected was found in the case of trisomy of chromosomes 3 in Mx-D (5/20), 5 in Imb (3/11), 7 in LC-F (7/20) and LC-D (13/54), and 12 in SmLy (13/41) (Table 2) when tested by the method of Brodeur et al.¹⁸ When the incidence of trisomy of given chromosomes in given histologic subtypes was compared with that in the entire CD lymphoma sample using the chi-square method,¹⁹ we observed a significant excess of chromosome 8 in Mx-F (4/16) and chromosome 21 in LC-D (9/54) as did Levine et al in their series.¹⁶ We additionally found a high incidence of trisomy 4 and X in LC-D (3/54 and 8/54, respectively), 5 in Imb (3/11), and 7 in LC-F (7/20) and LC-D (13/54).

Translocations. We identified 74 translocations in the 65 tumors analyzed. Of these, 42 (56.8%) belonged to three recurrent types, namely, t(8;14)(q24;q32) (10 cases), t(11;14)(q13;q32) (three cases), and t(14;18)(q32;q21) (29 cases). The remaining 32 (43.2%) were unique translocations (Table 3). The translocations t(14;18) and t(8;14) showed strong correlation with the grade of lymphoma; the former was seen in 76.5% (13/17) of low-grade tumors, 33.3% (9/27) of intermediate-grade tumors, and 14.3% (1/7) of high-grade tumors. Of the 8 intermediate-grade diffuse tumors that showed t(14;18); five were relapse specimens from patients in whom a previous biopsy had established the diagnosis of poorly differentiated lymphocytic lymphoma. The single high-grade tumor in our series with t(14;18) was a relapse tumor previously classified as LC-D. Interestingly, this tumor also showed t(8;22)(q24;q11), which is associated with high-grade tumors. In contrast, t(8;14) was absent in low-grade tumors and was present in 18.5% (5/27) of intermediate-grade and 42.9% (3/7) of high-grade tumors. Impressive associations between specific translocations and tumor subtypes were seen in the case of t(14;18) with SCC-F (9/10) and Mx-F (4/4), and t(8;14) with SNCC (3/3). We saw only three cases of t(11;14); two of these were in LNCC-D and one was in SCC-F (Table 3).

In the CD, t(14;18) was common in low- (49.6%) and intermediate- (24.0%) grade tumors and rare in high-grade tumors (3.6%; Table 3). Its incidence in the histologic subtypes was similar to that encountered in our series. It was absent from SmLy and was seen in 71% of SCC-F and 93.8% of Mx-F. Of 16 diffuse tumors in the CD that exhibited t(14;18), nine (56.3%) were reported to have evolved from a previous nodular lymphoma.

Table 2. Summary of Recurring Cytogenetic Changes in Histologic Subtypes of Malignant Lymphoma—CD*

Histologic Subtype	Tumors Studied (no.)	Cytogenetic Change			
		Trisomy	Translocation	Deletion	Breaks
SmLy	41	12	—	11q	14q
SCC-F	62	—	t(14;18)	6q	14q, 18q
Mx-F	16	8	t(14;18)	2q	2p, 14q, 18q
LC-F	20	7	t(14;18)	—	7p, 13q, 14q, 17q, 18q
SCC-D	6	—	—	8p, 20q	—
Mx-D	20	3	—	—	9p, 19q
LC-D	54	4, 7, 21, X	—	6q	1q, 4q, 6q, 9q, 14q, 18q
SNCC	12	—	t(8;14)	—	8q, 14q
Imb	11	5	—	3p, 5q, 6q	5p, 5q, 16q, 19p
Lyb	5	—	—	—	—

*See text for details.

Table 3. Frequency of Different Translocations in Histologic Subtypes of Malignant Lymphoma: Comparison of MSKCC Data With CD*

Histologic Subtype	Recurring Translocation						Unique Translocations		Total	
	t(8;14) (q24;q32)		t(11;14) (q13;q32)		t(14;18) (q32;q21)		MSKCC	CD	MSKCC	CD
	MSKCC	CD	MSKCC	CD	MSKCC	CD				
SmLy	0	1	0	4	0	0	0	21	0	26
SCC-F	0	0	1	1	9	44	3	38	13	83
Mx-F	0	0	0	0	4	15	0	16	4	31
LC-F	1	1	0	0	1	8	1	23	3	32
SCC-D	0	0	0	0	2	2	0	1	2	3
Mx-D	0	0	0	0	0	0	1	27	1	27
LC-D	4	9	2	4	6	14	15	58	29	85
SNCC	3	10	0	0	0	0	3	4	6	14
Imb	0	2	0	0	1	1	2	10	3	13
Lyb	0	0	0	0	0	0	0	5	0	5
Unclassified	2	2	0	0	6	7	7	9	13	18
Total	10	25	3	9	29	91	32	212	74	337

*Only translocations for which all breakpoints have been identified are included in the analysis.

In the CD, t(8;14)(q24;q32) similarly was the most common translocation in high-grade tumors (42.9%) and less so in intermediate-grade (10.0%). In the CD, t(8;14)(q24;q32) showed an 83.3% association with SNCC lymphomas. The third recurring translocation, t(11;14)(q13;q32), was absent in high-grade tumors in the CD, but was presented in low- and intermediate-grade tumors with nearly equal frequency (4.2% and 3.7%, respectively). In addition, four other translocations recurred at least twice each in the CD, but showed no correlation with histologic subtypes. These were t(3;14)(p21;q32), t(5;14)(q23;q32), t(11;14)(q23;q32), and t(14;19)(q32;q13).^{14,16}

Chromosome 1 was one of the participants in 18/32 unique translocations in our data and was the most frequent participant in these translocations. Regions 1p31-36 and 1q21-25 exhibited clustering of translocation breaks (seven and six times, respectively). In the CD, chromosome 1 was involved in 63/413 unique translocations. Of these region 1p31-36 was involved in 21 translocations and the region 1q21-25 was involved in 17 translocations. Thus, although

chromosome 1 seemed to be less frequently involved in translocation in the CD than in our series, when translocations affected chromosome 1, the frequency with which breaks clustered in the 1p31-36 and 1q21-25 was almost as high as in our series (60% v 70%).

Deletions. We identified chromosome deletions in 29 tumors and 1p and 6q were most frequently affected (Fig 2). The histologic groups SCC-D, LC-D, and Imb were frequently affected by deletion (75%, 52.6%, and 100%, respectively). Of the four tumors with del(1p), two were LNCC-D and of the three tumors with del(3p), two were Imb.

In the CD, deletions affected all chromosomes with the exception of chromosome 21 (Fig 2). Within histologic subtypes, 6q deletions were in excess in SCC-F, LC-D, and Imb while 11q deletions were in excess in SmLy ($P < 0.05$).¹⁸

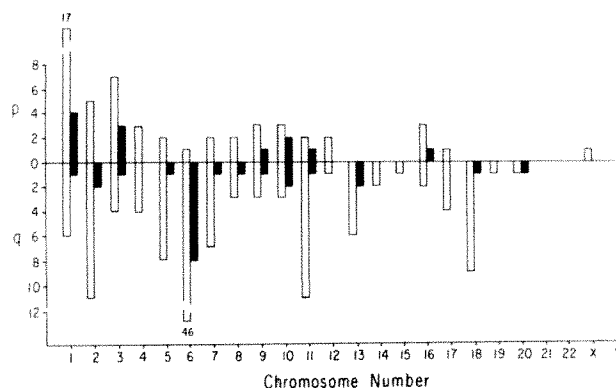


Fig 2. Deletions of chromosomes in malignant lymphoma. p and q are short and long arms, respectively. Overall, del(6q) in the MSKCC data and del(1p) and (6q) in the CD were significantly in excess. However, deletions of several chromosomes were significantly in excess in certain histologic subtypes (see text). □, Combined data; ■, MSKCC data.

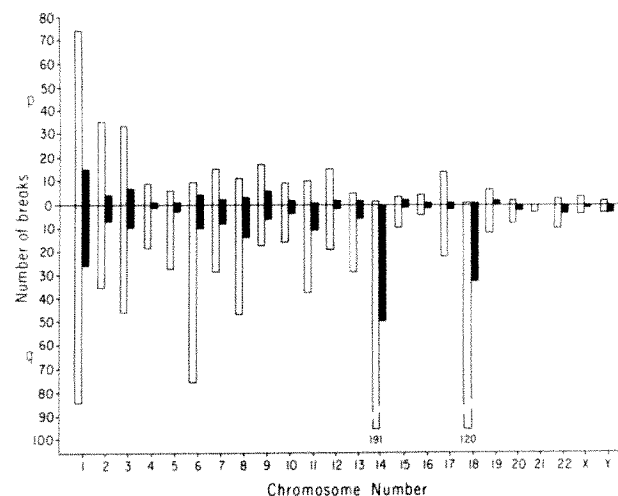


Fig 3. Chromosome breaks in malignant lymphoma. p and q are short and long arms, respectively. (Centromere breaks are not shown in this diagram; they were also not included in the analysis.) While the statistical significance of breaks in the MSKCC data was not tested, in the overall CD breaks in 1p, 1q, 6q, 14q, and 18q were significant. In addition, breaks in certain chromosomes were significantly in excess in certain histologic subgroups (see text). □, Combined data; ■, MSKCC data.

When the incidence of deletions in given chromosomes in given histologic subtypes was compared with that of the same deletion in the entire CD, the following significant deviations from expected were encountered: 2q in Mx-F, 3p and 5q in Imb, 6q in LC-D, 8p and 20q in SCC-D, and 11q in SmLy ($P < 0.05$)¹⁹ (Table 2).

Chromosome instability. We identified 270 breaks associated with specific as well as nonspecific rearrangements in our series (Fig 3). In the follicular tumors 66.7% of breaks were confined to chromosome 1, 14, and 18 while in the diffuse tumors 37.9% of breaks affected these three chromosomes. Chromosome 1 showed 15.2% of all breaks with clustering in regions 1p31-36 (12/14) and 1q21-24 (18/26). The following histologic subtypes exhibited recurrent chromosome 1 breaks: SCC-F (5/10), Mx-F (2/4), LC-D (7/19), and Imb (2/3). Breaks in the 6q21-23 region occurred in LC-D (3/19). Chromosome 7 showed 10 breaks, two in the short arm and eight in the long arm. Three of the 7q breaks were in one Imb. Chromosome 8 was most frequently affected in diffuse tumors and all breaks at 8q24 resulted in t(8;14). Breaks in chromosome 11 were clustered in the 11q13 region and were frequently seen in LC-D (5/19). Chromosome 14 showed 50 breaks (18.6% of total breaks) and 47 of them were at 14q32. Band 14q22 was affected in one SmLy and band 14q24 was affected in one Imb and in one unclassified tumor. Chromosome 18 showed 35 breaks of which 32 were at 18q21, and all of these were involved in t(14;18). Breaks in chromosomes 19, 20, 22, X, and Y were infrequent, although all three breaks encountered in chromosome 22 were at 22q11.

In the CD, a total of 1,211 breaks were enumerated for analysis (Fig 3). In the follicular tumors, 51.4% of breaks were confined to chromosomes 1, 14, and 18 while in diffuse tumors, 34% of breaks affected these same chromosomes. Chromosome 1 showed 13.4% of all breaks with clustering in regions 1p31-36 (52/71) and 1q21-24 (36/85), thus reflecting the clustering of translocation breaks in these sites stated above. Overall, a significant excess ($P < 0.05$) of breaks were seen in chromosome arms 1p, 1q, 6q, 14q, 18q, and 19q. Statistically significant associations ($P < 0.05$) were noted for breaks in chromosome 1 and lymphomas showing SCC-F and LNCC-D histology, breaks at 8q24 and SNCC lymphoma, breaks at 14q32 and SCC-F, Mx-F, and SNCC lymphomas, and breaks at 18q21 and SCC-F and Mx-F lymphomas. Additional significant correlations occurred in a number of histological subtypes and these are summarized in Table 2. Breaks in a number of chromosome arms were clustered in specific band regions, eg, 1p31-36, 1q21-32, 6q21-23, 8q24, 11q13, 14q32, 18q21, and 19q13.

DISCUSSION

Our studies presented here clearly show that recurrent cytogenetic changes characterize each of the histologic subtypes of malignant lymphoma in which more than one tumor has been studied; some of these correlations were previously established^{13,16} while others are recognized here for the first time.

The data set analyzed here represents the largest group of lymphoma karyotypes assembled so far and it shows that

cytogenetic findings are of diagnostic importance in NHL of B or null cell type (Table 2). While a variety of abnormalities have been identified, most have involved breaks at a limited number of bands. The finding of breaks at certain sites (eg, 8q24, 14q32, and 18q21) in a small cell neoplasm, especially one that arises in a nonlymphoid site, indicates that the lesion most probably is a lymphoma. The most important cytogenetic abnormalities in lymphomas appear to be a group of three recurring reciprocal translocations, eight trisomies, and seven deletions. Although highly significant associations between some of the abnormalities, especially the translocations, and histologic subtypes existed, the correlations were not unique and absolute, as is the case with most other tumor-associated specific chromosome changes.^{7,8} Thus, t(14;18) was very strongly associated with low-grade lymphomas and t(8;14) was associated with high-grade lymphomas. In addition, t(14;18) also was strongly associated with a nodular histology.

We have shown that breaks occurred nonrandomly in a number of chromosomal regions. Such breaks appeared to assume three distinct but recurring patterns: (1) Breaks that generate the specific translocations that are associated with specific histologic subtypes, namely, 8q24, 11q13, 14q32, and 18q21 (Table 3). These translocations bring about the juxtaposition of the cellular oncogenes *c-myc* (8q24),²⁰ *bcl-1* (11q13),²¹ and *bcl-2* (18q21)^{22,23} with the immunoglobulin heavy chain determinant (14q32). Translocation-mediated deregulation of *c-myc* has been demonstrated^{24,25} and it is highly likely that the other two genes are similarly deregulated by the translocations. Translocations affecting these three sites together characterized 47.3% of cytogenetically abnormal tumors in the CD. Furthermore, in several tumors in this data set they represented the sole cytogenetic abnormality suggesting that the gene deregulation that these translocations bring about plays a primary role in the development of lymphoma. (2) Breaks that generate unique translocations that are not correlated with histologic subtypes (Table 3). The best examples of this class of breaks are the chromosome 1 sites 1p34-36 and 1q22-24. These translocations never were encountered as the sole chromosome abnormality but were always seen as additional abnormalities. The cellular oncogenes *c-src*²⁶ and *c-ski*²⁷ have been mapped to these two sites, respectively. Perturbations affecting these sites are common not only in lymphomas but also in tumors of diverse cell types²⁷ suggesting that deregulation of these oncogenes may be associated with nonspecific tumor progression. (3) Breaks that generate deletions of chromosomes 1p, 2q, 3p, 5q, 6q, 8p, 11q, and 20q that correlate with histologic subtypes (Table 2). Hemizygosy or homozygosity for the nondeleted chromosome followed by reduplication of the remaining chromosome has been shown to be an important genetic event in the etiology of retinoblastoma²⁸ and Wilms' tumor.²⁹ Some of the sites of recurring deletions seen in our analysis of lymphoma also are the sites of cellular oncogenes, for example, *c-src* (1p34-36) and *bcl-1* (11q13). Other sites of deletion that we identified may turn out to be the sites of important genes yet to be discovered. Hemizygosy-homozygosity of the sites may act as a step in lymphoma development. Thus, the cytogenetic analysis presented here

identified for the first time distinct levels of genetic alteration associated with lymphoma development thereby providing new leads for further molecular analysis.

Histologic parameters that relate to prognosis continue to be defined,³⁰ and may result in revision of current classification of lymphomas. The prognostic implications of cytogenetic changes in lymphoma remain largely as unanswered questions. One immediate question relates to diffuse tumors that show t(14;18). We have established that a proportion of these had a previous nodular histology. Whether diffuse lymphomas that represent transformation from a previous nodular histology would have a better prognosis than those

that lack the t(14;18) needs to be determined. As more data from prospective series such as the ones analyzed in this paper become available, further definition of the cytogenetic correlations noted here as well as recognition of new correlations can be expected.

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We are grateful to Dr Clara D. Bloomfield for a preprint of the article by Levine et al¹⁶ that summarized the cytogenetic analysis of the series of lymphomas studied by them and to Dr Jorge J. Yunis for providing the full karyotypes of tumors reported in 1982.¹³

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Identification and Characterization of a Differentiation-Specific Antigen on Normal and Malignant Murine Erythroid Cells

By E. Rena Bacon and Arthur J. Sytkowski

Rauscher murine erythroleukemia cells grow continuously in vitro and undergo terminal differentiation in response to the physiological inducer erythropoietin. In the course of this developmental process they express many erythroid-specific markers. In order to investigate the expression of cell surface determinants during Rauscher cell differentiation we generated monoclonal antibodies to uninduced cells. Using an anti-Rauscher cell monoclonal antibody, we have identified a cell surface determinant, designated ERY-1, that is present on normal murine erythroid cells.

MURINE and human erythroleukemia cell lines have been studied extensively as model systems of erythroid commitment and differentiation. The Rauscher murine erythroleukemia cell line is particularly suitable for such investigation because of the ability of these cells to differentiate in response to the physiologic inducer erythropoietin as well as to nonphysiologic inducers, eg, dimethyl sulfoxide (DMSO).^{1,2} The cell line was derived originally from Rauscher virus-infected DBA mice and was cloned to establish more homogeneous populations.² Rauscher cells resemble immature erythroblasts morphologically, and they differentiate into hemoglobin-containing erythroid cells after induction. They synthesize all three adult murine globins—alpha, beta major, and beta minor.³ Erythropoietin induction also results in increased levels of beta-adrenergic receptors⁴ and membrane spectrin (Bacon ER, unpublished observations). While the erythroid nature of Rauscher cells seems certain, the stage of differentiation of the uninduced cells relative to normal erythropoietic progenitors has not yet been determined clearly. Some experimental evidence suggests that noninduced Rauscher cells may be at a differentiation stage similar to that of the normal erythroid progenitor BFU-E. Rauscher cells growing in semisolid medium can exhibit a rather diffuse colony morphology reminiscent of the BFU-E-derived colony. Moreover, their erythropoietin dose-response relationship is remarkably similar to that of the normal mouse BFU-E.^{2,3}

In order to examine the process of Rauscher cell differentiation further, we chose to investigate changes in the expression of cell surface determinants that occur during maturation and to compare these changes to those that occur during normal erythropoiesis. Therefore, we generated monoclonal antibodies against noninduced (and thus immature) Rauscher cells. We anticipated that the antibodies would also recognize bone marrow erythroid cells, including progenitors. We describe here the identification and characterization of an erythroid-specific surface antigen, designated ERY-1, which is expressed during certain stages of maturation of normal erythroid progenitors and of Rauscher cells.

MATERIALS AND METHODS

Production of Monoclonal Antibodies

Male Lewis rats were immunized intraperitoneally with 3×10^7 Rauscher cells (clone 404) at monthly intervals. Sera from immunized rats were assayed for anti-Rauscher cell antibodies by

This determinant is apparently absent from the early progenitor BFU-E, but is present on the more mature progenitors CFU-E and CFC-E. It disappears during erythroid maturation and is absent from the mature erythrocyte. This pattern of ERY-1 expression is exhibited with remarkable fidelity during the erythropoietin-induced differentiation of Rauscher cells. Such differentiation-specific expression of the ERY-1 determinant suggests that it may play a functional role in erythropoiesis.

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enzyme-linked immunosorbent assay (ELISA; see below), and an animal with a high anti-Rauscher cell antibody titer was identified. Four days prior to the fusion, the animal selected was boosted intravenously with 5×10^7 cells. At the time of the fusion the animal was killed by CO₂ asphyxiation, and the spleen was removed. The fusion protocol was modified from the method of Kennett.⁵ Briefly, 7.5×10^7 spleen cells and 1.5×10^7 P3X63Ag8653 myeloma cells (Institute for Medical Research, Camden, NJ) were fused in the presence of 0.3 mL of 30% polyethylene glycol 1500 (PEG; BDH, Carle Place, NY). The cells were incubated at 37 °C for two minutes and then centrifuged at $260 \times g$ at 25 °C for six minutes. The supernatant was aspirated, and the cell pellet was diluted slowly with 30 mL of HY-HT medium (70% Dulbecco's MEM, 20% fetal calf serum, 10% NCTC-109 [GIBCO, Grand Island, NY], supplemented with 1.6×10^{-5} mol/L glucose, 2 mmol/L glutamine, 1 mmol/L oxaloacetic acid, 0.5 mmol/L pyruvic acid, 0.2 U/mL insulin, 1×10^{-4} mol/L hypoxanthine, 1.6×10^{-5} mol/L thymidine, and 2.5×10^{-5} mol/L 2-mercaptoethanol [Sigma, St Louis, Mo]). The cells were incubated overnight in a 150 mm plastic petri dish in 10% CO₂ at 37 °C. The next day, the cells were centrifuged and resuspended in 30 mL HY-HT medium containing 4×10^{-6} mol/L aminopterin (HAT medium). The cells were plated into six 96 well Costar (Cambridge, MA) microculture dishes on a feeder layer of human fibroblasts and incubated in a humidified incubator in 10% CO₂ at 37 °C. The cells were fed weekly with 50 uL of HAT medium per well. The supernatants from the wells containing macroscopic hybridoma colonies were screened by ELISA for antibody to Rauscher cells, to syngeneic adult murine liver cells, and to thymocytes. Colonies whose supernatants contained antibodies to Rauscher (R) cells but not to adult liver cells (L) or thymocytes (T) (R⁺L⁻T⁻ antibodies) were selected for further investigation and cloned. One such antibody was found to recognize a determinant found on erythroid precursors (see Results).

Assays for Reactivity of Monoclonal Antibodies

Enzyme-linked immunosorbent assay (ELISA). Rauscher cells, adult murine liver cells, or thymocytes were centrifuged for five

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minutes at $700 \times g$ and fixed with 0.05% glutaraldehyde into 96 well flexible plastic round bottom wells (Dynatech, Alexandria, Va) at a concentration of 10^6 cells/mL (0.1 mL/well). The wells were washed twice and blocked with Dulbecco's phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA; Miles Scientific, Naperville, Ill) for one hour. Supernatants from the hybridoma wells were added to each of the three test cell plates (Rauscher cells, liver cells, thymocytes) and incubated for 45 minutes at 4°C . The ELISA plates were washed with the PBS-BSA buffer and incubated with 50 μL /well of 1:1000 horseradish peroxidase-conjugated rabbit antirat IgG antibody (Cooper Biomedical, Malvern, Pa) for 45 minutes at 4°C . The plates were washed, and 50 μL of 6 mmol/L O-phenylenediamine/0.01% H_2O_2 (Sigma) substrate reagent was added to each well. The reaction was stopped by adding 2N HCl, and the resulting chromophore was measured at 490 nm with an EIA reader (Bio-Tek, Burlington, Vt).

Immunofluorescence. Antibody binding to Rauscher cells or to DBA/2J murine bone marrow cells, splenocytes, thymocytes, neutrophils, peritoneal macrophages, and platelets was assessed by fluorescence flow cytometry (FFC) with an Ortho Cytofluorograf System 50 interfaced with 2150 Ortho computer. Cells (5×10^6) were incubated with 100 μL of $\text{R}^+\text{L}^-\text{T}^-$ hybridoma supernatant for 30 minutes at 4°C . They were washed three times with PBS and then incubated with 100 μL of 1:25 fluorescein isothiocyanate-conjugated rabbit antirat IgG (Cooper Biomedical). The cells were washed and resuspended in 25 mmol/L HEPES-buffered MEM, 2% fetal calf serum, pH 7.3, for fluorescence analysis and sorting. Sorted cells were collected into 35 mm petri dishes containing 1 mL of heat-inactivated fetal calf serum. For subsequent morphologic examination, the cells were washed and resuspended in fetal calf serum, cytocentrifuged onto glass slides (Shandon-Southern Cytospin SCA-0030, Sewickley, PA) and stained with Wright's Giemsa (Harleco). For differential counts, at least 500 cells per slide were examined. Portions of cells were collected under sterile conditions for in vitro progenitor assays. Neutrophils were obtained from the peritoneal exudates of male mice three hours after a single injection of calcium caseinate.⁶ Microscopic examination of the cells fixed to glass slides and stained with Wright's stain confirmed that 80% to 90% of the cells were neutrophils. Peritoneal macrophages were harvested by peritoneal lavage of anesthetized but otherwise untreated mice.

In Vitro Hematopoietic Progenitor Assays

BFU-E, CFU-E, and CFC-E culture. The sorted cell populations were analyzed for BFU-E, CFU-E, and CFC-E⁷ in plasma clot culture.⁸ Bone marrow cells treated with antibody were separated by FFC into two populations, those exhibiting moderate-to-high fluorescence (positive) and those with low fluorescence (negative). Antibody-treated, unsorted cells served as the control for these experiments. CFU-E/CFC-E cultures were grown for 48 hours in the presence of 0.25 U erythropoietin/mL, while BFU-E cultures were grown for seven days in the presence of 3 U erythropoietin/mL (Connaught, Willowdale, Ontario). At specified times, the clots were transferred to microscope slides, fixed with glutaraldehyde, stained for hemoglobin with dimethoxybenzidine, and counterstained with hematoxylin. The slides were scored for both erythroid colonies from CFU-E (8 or more cells) and for erythroid clusters from CFC-E (3 to 7 cells).⁷ The seven-day cultures were scored for BFU-E-derived colonies consisting of three or more subcolonies.

CFU-C culture. The sorted populations were assayed for CFU-C granulocytic progenitor growth as described by Marchal and Milon.⁹ Bone marrow cells were plated in 96 well microtiter dishes at six concentrations, ranging from 400 cells to 12.5 cells/well (60 wells for each cell concentration), in DMEM containing 20% fetal calf

serum, 5×10^{-5} mol/L 2-mercaptoethanol in the presence of colony-stimulating factor (CSF) derived from both sera- and lung-conditioned media from endotoxin-treated mice.¹⁰ The cells were incubated for seven days in 10% CO_2 at 37°C . Cytocentrifuge preparations from the wells containing colonies demonstrated complete myeloid cell differentiation in this culture system, confirming the identity of the colonies as derived from CFU-C. Limiting dilution analysis permitted the determination of the number of precursor cells in the population. In this assay system diluting the concentration of cells plated increases the incidence of wells with no colonies. The percentage of wells lacking colonies for each cellular concentration plated was then plotted semilogarithmically. According to the Poisson distribution and assuming a random and independent distribution of progenitors throughout the wells, the concentration of cells plated in which 37% of the wells are negative for CFU-C colony growth is the cell concentration that contains one CFU-C.¹¹

Phenylhydrazine anemia. A phenylhydrazine-induced hemolytic anemia was generated in DBA/2J mice using a modification of the method of Spivak et al.¹² Mice were injected intraperitoneally with 0.04 mg phenylhydrazine (Sigma) per gram animal weight at 9 AM and 3 PM on day 1 and 9 AM on day 2. Blood was obtained on day 5 from the retro-orbital venous plexus.

Erythropoietin induction of Rauscher cells. Rauscher cells were induced with 2 U of erythropoietin/mL for specified time intervals ranging from five minutes to six days. Erythropoietin was prepared in our laboratory from the urine of anemic human donors and had a specific activity of 1050 U/mg. The cells were harvested, washed in PBS, and ERY-1 was measured by FFC. The changes in antigen density were quantitated by converting the median fluorescence intensity of the experimental samples from a log scale to linear fluorescence intensity units.^{13,14}

Western blot analysis of ERY-1 antigen. Whole cell lysates and plasma membrane preparations of logarithmically growing Rauscher cells were subjected to discontinuous polyacrylamide gel electrophoresis through 10% gels. The proteins were then transferred to nitrocellulose paper electrophoretically¹⁵ and remaining protein binding sites were blocked with a solution of 3% bovine serum albumin in 25 mmol/L Tris-HCl, 500 mmol/L NaCl, pH 7.5. The nitrocellulose was incubated for two hours with dilutions of ERY-1 monoclonal antibody (MAb) ascites (Deca-Probe, Hoefer Scientific, San Francisco), washed, and then incubated with 1:1000 horseradish peroxidase-conjugated goat antirat IgG. Bound antibody was detected with 4-chloro-1-naphthol and H_2O_2 .

RESULTS

Binding of Monoclonal Antibody ERY-1 to Rauscher Cells and to Normal Marrow Cells

Numerous supernatants from the fusion contained antibodies that bound to Rauscher cells but not to liver cells or thymocytes in the screening ELISA ($\text{R}^+\text{L}^-\text{T}^-$ antibodies; see Materials and Methods). The binding of these antibodies to Rauscher cells and to normal marrow cells was examined by FFC. The Rauscher cell-binding profile of one of these antibodies, designated ERY-1, is shown in Fig 1. Greater than 80% of Rauscher cells bound MAb ERY-1 compared to the control rat IgG. In contrast, only 26% of Friend murine erythroleukemia cells bound MAb ERY-1 (not shown), and this was at far lower fluorescence intensities than the Rauscher cells. Human K-562 cells did not bind MAb ERY-1.

The initial flow cytometric analysis of MAb ERY-1 binding showed that a population of cells within normal

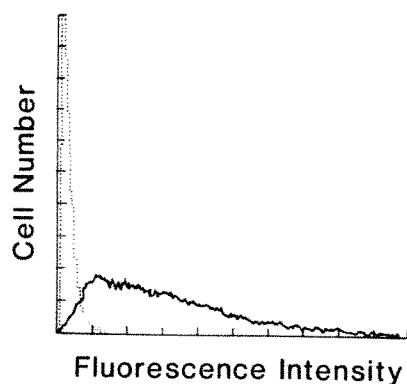


Fig 1. Fluorescence flow cytometry profile of Rauscher cells analyzed with MAb ERY-1 (solid line). The dotted line represents normal rat IgG control.

murine bone marrow bound the antibody (Fig 2A). Those cells that exhibited MAb ERY-1 binding were a continuous rather than discrete population whose fluorescence distribution was a broad shoulder of gradually increasing fluorescence intensity when compared to the control cell population that was incubated with irrelevant rat IgG (Fig 2A). This fluorescence distribution was different from that obtained when an antimyeloid monoclonal antibody, MAb MYELO-2, was used (Fig 2B). In this case a discrete population of cells was well separated from the control (and nonreactive) population.

Using MAb ERY-1, we sorted normal bone marrow cells

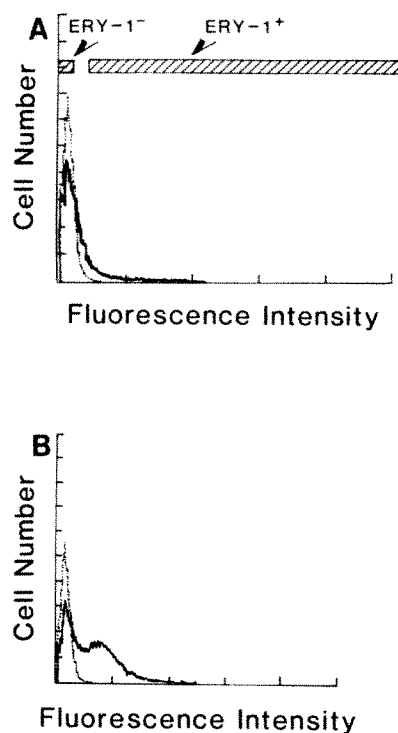


Fig 2. Fluorescence flow cytometry profiles of bone marrow cells sorted with anti-Rauscher cell monoclonal antibodies ERY-1 (A) and MYELO-2 (B). The dotted lines are normal rat IgG controls.

Table 1. Binding of ERY-1 Monoclonal Antibody to Normal Murine Bone Marrow Cells Assessed by Fluorescence Flow Cytometry

Population	Cell Type (%)		
	Erythroid	Lymphoid	Myeloid
ERY-1 ⁺	44	17	39
ERY-1 ⁻	0	14	86
Unsorted	16	22	62

into "positive" (ERY-1⁺) and "negative" (ERY-1⁻) populations (see Fig 2A for sorting gates) and examined them morphologically after spinning onto glass slides, fixing, and staining (Table 1). The ERY-1⁺ population consisted of 44% erythroid cells, 17% lymphoid cells, and 39% myeloid cells. This was in contrast to the ERY-1⁻ population, which contained 0% erythroid cells, 14% lymphoid cells, and 86% myeloid cells. These data suggested to us that MAb ERY-1 recognized a determinant that was at least predominantly erythroid and prompted further investigation using highly enriched populations of hematopoietic cells.

Binding of Monoclonal Antibody ERY-1 to Enriched Populations of Hematopoietic Cells

Further confirmation of the erythroid nature of the ERY-1 antigen was sought by examining the binding of MAb ERY-1 to fetal and adult liver cells. Liver erythropoiesis is quite brisk in the 12- to 13-day-old murine fetus. However, it begins to decline markedly at day 14 and has essentially ceased at the time of birth. Using fluorescence flow cytometric analyses, we found that the 13-day murine fetal liver contained 70% ERY-1⁺ cells (Table 2). This value decreased to 35% ERY-1⁺ at day 14 and, finally, to a total absence of ERY-1⁺ cells in the adult. Thus, the pattern of MAb ERY-1 binding to murine fetal liver cells paralleled that of hepatic erythropoiesis, supporting the identification of the ERY-1 determinant as an erythroid antigen.

In contrast, MAb ERY-1 did not bind to enriched populations of neutrophils, splenic lymphocytes (predominantly B cells), thymocytes, peritoneal macrophages, or platelets (Table 2). These results provided strong evidence that the ERY-1 determinant is restricted to the erythroid lineage and that the presence of nonerythroid cells in the ERY-1⁺ population obtained after sorting normal bone marrow (Table 1) was due to the gate settings of the Cytofluorograf.

Table 2. Binding of ERY-1 Monoclonal Antibody to Murine Hematopoietic Cells

Cell Type	ERY-1 ⁺ Cells (%)
Fetal liver	
13 day	70
14 day	35
Adult liver	0
Neutrophils	<1
Lymphocytes, spleen	<1
Thymocytes	<1
Macrophages, peritoneum	<1
Platelets	<1

Identification of ERY-1⁺ Cells in the Bone Marrow, Spleen, and Peripheral Blood During Erythropoietic Stress

Our initial flow cytometric and morphological examination of MAb ERY-1 binding to normal bone marrow indicated that the monoclonal antibody bound to erythroid precursors. Therefore, conditions of erythropoietic stress that result in higher frequencies of erythroid precursors should cause an increase in the relative number of ERY-1⁺ cells. We compared the percentages of ERY-1⁺ cells present in the bone marrow, spleen, and peripheral blood of normal animals to those in animals with phenylhydrazine-induced hemolytic anemia (Table 3). The number of ERY-1⁺ cells in the normal bone marrow of three mice tested ranged from 12% to 19%. This value increased to 38% during phenylhydrazine anemia. Similarly, the number of ERY-1⁺ cells in the spleen rose from less than 1% in the normal spleen to 32% in the erythropoietic spleen of phenylhydrazine treated animals. Lastly, the peripheral blood of normal animals contained 0% ERY-1⁺ cells. This value rose markedly to 40% during phenylhydrazine anemia.

Binding of Monoclonal Antibody ERY-1 to Progenitor Cells

The data presented thus far indicated that the ERY-1 antigen is present on developing erythroid precursors but that it disappears sometime before erythroid maturation is complete. Since this pattern implied that the expression of ERY-1 is specific for certain states of erythroid differentiation, we considered it important to determine the distribution of this determinant among various progenitor cells. The analysis was performed as follows. Normal bone marrow cells were reacted with ERY-1 monoclonal antibody and separated into ERY-1⁺ and ERY-1⁻ populations by FFC. Approximately 15% to 20% of the normal cells were designated ERY-1⁺. Sorted populations of cells that were either ERY-1⁻ or moderately to strongly ERY-1⁺ were collected and assayed *in vitro* for BFU-E, CFU-E, and CFC-E, and for granulocyte progenitors (CFU-C) (Table 4).

The most primitive of the erythroid progenitors, BFU-E, was completely absent from the ERY-1⁺ population and was present in the ERY-1⁻ population. This was in marked contrast to the more mature progenitor CFU-E which was found almost exclusively (98%) in the ERY-1⁺ population. This appearance of the ERY-1 antigen on the CFU-E was

Table 3. Identification of ERY-1⁺ Cells in Bone Marrow, Spleen, and Peripheral Blood of Normal and Phenylhydrazine-Treated Animals

Cells	ERY-1 ⁺ Cells (%)
Bone Marrow	
Normal	12, 19, 17*
Phenylhydrazine	38
Spleen	
Normal	<1
Phenylhydrazine	32
Peripheral Blood	
Normal	0
Phenylhydrazine	40

*Three separate determinations.

Table 4. Binding of ERY-1 Monoclonal Antibody to Progenitor Cells

Progenitor	Sorted Population*	
	ERY-1 ⁺	ERY-1 ⁻
BFU-E	0	12
CFU-E	240	6
CFC-E	85	0
CFU-C	83	555

*Progenitors per 10⁵ cells.

maintained into the most mature progenitor population, the CFC-E, all of which were ERY-1⁺. As expected, the granulocyte progenitors, CFU-C, were found highly concentrated in the ERY-1⁻ population. Thus, detectable ERY-1 expression appears to begin between the BFU-E and CFU-E stages of erythroid progenitor differentiation and continues through erythroid progenitor development. The determinant is found on identifiable precursors but disappears before final erythrocyte development (Fig 3).

ERY-1 Expression During Rauscher Cell Differentiation

The pattern of ERY-1 antigen expression occurring during normal erythroid development was reflected during the differentiation of Rauscher cells. As shown in Fig 4, the density of ERY-1 antigen was slightly higher after six hours and doubled within the first day of erythropoietin induction, analogous to the increase found as BFU-E develop into CFU-E. This was followed by a reduction to 137% of the starting density after two days and a dramatic decrease to less than 7% of maximum after six days of induction with erythropoietin. This pattern of ERY-1 expression, which was identified in three replicate experiments, strongly resembles the pattern found during normal erythropoiesis, further confirming the similarity of Rauscher cell differentiation to normal bone marrow erythroid development. In similar experiments using dimethyl sulfoxide induction, no initial increase in ERY-1 was detected. As DMSO induction was continued, however, ERY-1 expression diminished correspondingly and was less than 10% after six days.

Western Blot Analysis of ERY-1

Rauscher cell plasma membrane preparations and whole cell lysates were subjected to SDS gel electrophoresis and Western blot analysis in an effort to begin a biochemical characterization of the ERY-1 antigenic determinant. However, despite repeated efforts, no specific binding of the MAb to any protein bands was found. This strongly suggests that the epitope identified by the antibody on the native antigen is altered upon denaturation with SDS.

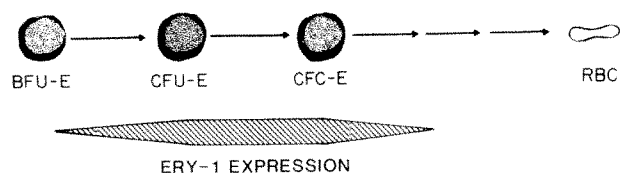


Fig 3. Model of ERY-1 antigen expression during normal erythropoiesis.

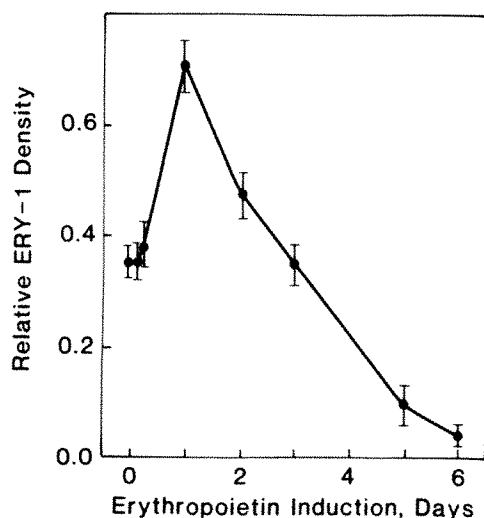


Fig 4. Effect of erythropoietin induction on Rauscher cell ERY-1 membrane antigen density. Units of antigen density are linear fluorescence units as volts converted from the log scale.^{13,14} The fluorescence obtained using equivalent concentrations of irrelevant rat IgG was less than 0.05 units at all time points and has been subtracted from the ERY-1 values. The points represent the mean $\pm$ SE of three replicate experiments.

DISCUSSION

In the present study we have employed the continuous cell line Rauscher murine erythroleukemia as immunogen and have generated a monoclonal antibody that recognizes an antigenic determinant, designated ERY-1, present on normal erythroid progenitors and precursors. The data indicate that the ERY-1 antigen is restricted to the erythroid lineage and is expressed only during certain stages of erythropoiesis.

Initial FFC analyses (Fig 2A) showed that the population of bone marrow cells expressing ERY-1 was continuous with the negative population rather than separated discretely (Fig 2B). Hence, the fluorescence intensities employed to discriminate "positive" from "negative" cells were necessarily somewhat arbitrary. The consequences of this partial overlap of populations is seen in Table 1. Thus, although no erythroid precursors were found in the ERY-1⁻ population (ie, all erythroid precursors were ERY-1⁺), the ERY-1⁺ population in this analysis was not exclusively erythroid. Although an alternate explanation for this observation is that a portion of both myeloid and lymphoid cells actually expresses the ERY-1 determinant, this seems quite unlikely, since the ERY-1 determinant was not found on highly enriched populations of these and other nonerythroid hematopoietic cells (Table 2).

ERY-1 antigen was not found on the early erythroid

progenitor, BFU-E. In this regard it is important to note that the lower limit of detection of cell surface determinants using conventional FFC, such as that employed in this study, is approximately 10,000 molecules per cell. Thus, the designation of BFU-E as ERY-1⁻ must be considered as relative rather than absolute. Nonetheless, ERY-1 expression is clearly increased markedly in the CFU-E and CFC-E and decreases thereafter during precursor maturation. Importantly, this pattern of ERY-1 expression was exhibited with remarkable fidelity during erythropoietin-induced Rauscher cell differentiation, supporting the use of these cells as a model system for the study of erythropoiesis.

Monoclonal antibodies that recognize surface determinants restricted to immature mammalian erythroid cells have been reported previously. Yokochi et al described two antibodies that bind to immature human erythroid cells at different stages of differentiation.¹⁶ A third MAb, reported recently from this same laboratory, binds to CFU-E, erythroblasts, and mature erythrocytes.¹⁷ Tonevitsky et al have developed a rat MAb that recognizes a 69 kD antigen found on murine nucleated red cells and reticulocytes.¹⁸ Although many other MAbs that bind to erythroid cells have been described, strict lineage specificity is quite unusual.

At present, the identity and function of the ERY-1 antigen is unknown. It does not appear to be associated with the transferrin receptor since it exists at a very low density on Friend cells which are known to express greater than 10⁵ copies of the transferrin receptor per cell.¹⁹ Moreover, the absence or presence of excess transferrin has no effect on the binding of MAb ERY-1 to Rauscher cells (data not shown). Since the pattern of ERY-1 expression during differentiation of erythroid progenitors resembles the pattern of erythropoietin sensitivity, it is possible that the ERY-1 antibody recognizes the erythropoietin receptor or a macromolecule associated with it. The addition of ERY-1 antibody to Rauscher cell cultures results in striking cell clustering but does not interfere with the ability of the cells to grow or to respond to erythropoietin (data not shown). Nevertheless, the antibody could recognize a portion of the receptor remote from the hormone-binding domain. Another possibility to be considered is a relationship between the ERY-1 determinant and the fibronectin binding sites found on erythroleukemia cells and on immature, normal erythroid cells.^{20,21} ERY-1 expression during normal and Rauscher cell erythropoiesis is remarkably similar to that reported for these sites. Finally, the similarity of ERY-1 to EP-1, a determinant found on human erythroid cells,¹⁶ must be noted. Establishing the identity and function of ERY-1 and other related differentiation-specific antigens may be pivotal in elucidating the processes of erythropoietic commitment and differentiation.

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Hydroxyurea-Induced Augmentation of Fetal Hemoglobin Production in Patients With Sick Cell Anemia

By Samuel Charache, George J. Dover, Martha A. Moyer, and Jay W. Moore

Five patients with sickle cell anemia were treated with hydroxyurea (HU), in hopes of augmenting their production of fetal hemoglobin. Laboratory responses in two patients treated for more than 2 years were encouraging and there were suggestions of clinical improvement. Long-term HU therapy should be considered for severely affected adults with sickle cell anemia who are willing to accept what is probably a small risk of carcinogenesis. Preliminary chromosomal analysis and knowledge of the clastogenic prop-

erties of HU suggest that conception and pregnancy should be avoided. Pharmacokinetic studies will probably be necessary to adjust individual dosage schedules so that cytotoxicity is avoided. F cell responses can be seen in 2 to 3 weeks if the HU dose is optimal, but establishment of a large number of F cells in the circulation may take a month or longer.

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IN 1948 Janet Watson reported that the red cells of newborns with sickle cell anemia did not sickle and suggested that inhibition of sickling was due to the presence of fetal hemoglobin (Hb F).¹ Discovery of pancellular hereditary persistence of fetal hemoglobin (HPFH) and its interaction with sickle cell trait (S/HPFH) provided clinical evidence for inhibition of sickling by Hb F,² and the studies of the Singers,³ Bertles et al,⁴ Nagel et al,⁵ and Goldberg et al⁶ provided explanations for inhibition at a molecular level.

In patients, it has been difficult to demonstrate a beneficial effect of increased Hb F levels on severity of illness, in no small part because of difficulties in defining severity. Increased longevity^{7,8} in adults, decreased sequestration crises in children,⁹ and decreases in certain well-defined events¹⁰ have been associated with increased Hb F, as have the milder courses of Saudi Arabians with the disease.^{11,12}

Much effort was devoted to attempts at therapeutic exploitation of these findings,¹³ but it was not until DeSimone et al showed that administration of 5-azacytidine (AzaC) to anemic baboons caused remarkable increases in Hb F production¹⁴ that clinical studies became possible. Cast in contemporary terms, but based on much older work,¹⁵⁻¹⁷ the goals of such studies were to raise the proportion of Hb F-containing cells (F cells) and the amount of Hb F/F cells, and compare these increases with decreases in hemolysis and the severity or frequency of vaso-occlusive crises.

We¹⁸ and others¹⁹ showed that AzaC increased Hb F levels in humans, and we gave preliminary evidence that it might have therapeutic benefit when used on a long-term basis. Methods were developed to permit its oral administration, a necessary prerequisite if it was to be used on a long-term basis,²⁰ but enthusiasm for use of AzaC was chilled by early^{21,22} and more recent²³ reports that the drug was probably a carcinogen in laboratory animals.

The mechanism of action of AzaC on Hb F synthesis is unclear.^{24,25} Three hypotheses have been advanced: (1) derepression due to hypomethylation of cytosine residues,^{18,19} (2) "recruitment" of early red cell precursors during recovery from drug-induced marrow suppression,²⁶ and (3) direct "reprogramming" of relatively late precursors by a direct effect of the drug.²⁷ The debate is still in progress, but the recruitment hypothesis led to use of hydroxyurea (HU) for augmentation of Hb F synthesis.²⁸⁻³¹ HU is readily available and easily administered; although reported to be noncarcinogenic³² in a limited study, it is a mutagen³³ and teratogen.^{34,35}

We report here our experience at The Johns Hopkins Hospital with long-term use of HU for treatment of patients with sickle cell anemia. Questions discussed include dosage regimens, hematologic effects, clinical responses, and untoward effects of the drug. Intensive observation of two patients for more than 2 years permits speculation on the number of F cells and proportion of Hb F needed to ameliorate crises and the role of dense red cells in crises, and has led to suggestions for future trials of the drug.

MATERIALS AND METHODS

Patients were considered for admission to the study only if they had failed or could not continue any other means of therapy and were seriously disabled by their disease. They were informed of potential carcinogenesis, and consent was not obtained until both the patient and a friend or relative had attended several sessions at which potential risks and benefits of treatment and obligations of investigator and research subject were described in detail. The consent form and the process of obtaining consent were approved by The Johns Hopkins Committee on Clinical Investigation.

Capsules of HU (Hydrea, Squibb, Princeton, NJ) were purchased at a pharmacy; no attempt was made to "blind" either patient or investigator. Baseline studies and initiation of therapy were done in the Inpatient Research Center; thereafter, patients were followed at 1- to 4-week intervals in the Outpatient Research Center. Patient H was followed up by Drs. P. Roberts and P. Milner (Medical College of Georgia, Augusta), and samples were sent by overnight express mail.

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Blood counts were done by a Coulter S+IV (Coulter Electronics, Hialeah, FL), reticulocytes were counted using thioflavin T and an EPICS-C flow cytometer (Coulter Electronics),³⁵ and Hb F in hemolysates was measured by alkali denaturation. Proportion of F cells (red cells that contain Hb F) and F reticulocytes (reticulocytes that contain Hb F) were assayed by previously described methods³⁶; the amount of Hb F/F cell was calculated from the Coulter mean corpuscular hemoglobin (MCH), the hemolysate Hb F level, and the proportion of F cells (Table 1); the calculation was validated by independent immunoprecipitation reactions in some instances.³⁷ Because MCH is the same in F and non-F cells,³⁸ Hb S/F cell and S/S cell were readily calculated. The proportion of dense red cells ($d > 1.120$, mean corpuscular hemoglobin concentration [MCHC] > 37 g/dL) and median corpuscular hemoglobin concentration were estimated after centrifugation through mixtures of phthalate esters of varying density.³⁹ Blood HU levels were measured at intervals after a test dose of 25 mg/kg (to the nearest 500 mg), using the method of Fabricius and Rajewsky⁴⁰ as modified by Belt et al.⁴¹ DNA haplotypes associated with the γ - δ - β gene cluster were determined in the laboratory of Dr S. Antonarakis (Johns Hopkins University School of Medicine, Baltimore).⁴² For chromosomal analysis, lymphocytes from peripheral blood were stimulated to divide with phytohemagglutinin and cultured for 72 hours in Eagle's minimum essential medium supplemented with 13% fetal calf serum (GIBCO, Grand Island, NY). Cells were arrested in metaphase with demecolcine, and a minimum of 30 G-banded chromosomes were counted and analyzed (without knowledge of the patient's identity) for structural rearrangements and aberrations.⁴³

When patients developed pain, they were treated at home with oxycodone and acetaminophen capsules; if pain could not be managed, they came to the emergency department. A decision whether or not they required admission was made within 12 hours by the staff there, with no advice or input from the investigators; decisions regarding discharge were made by the patient. Patients often asked about their hemoglobin or hematocrit levels but were not aware of Hb F, F reticulocyte, or F cell values. All patients were told to take 1 mg folic acid per day.

Patients

Responses of patients A and C to AzaC were described earlier¹⁸ and their and patient E's initial responses to HU have also been described.³¹ Patient A is now 33 years old and has transfusion-related hemochromatosis and diffuse but asymptomatic bone disease. Patient C is now 47; he has severe bone disease and early renal disease and has suffered two strokes. Patient E is a 31-year-old woman who has consumed large amounts of narcotics because of recurrent intractable abdominal pain; she was referred by Dr T.

Gabuzda (Philadelphia). Patient F is a 25-year-old woman who had unremitting ankle ulcers and severe anemia; she could not be transfused because of prior alloimmunization. Patient G, 31 years old, was referred by Dr S. Glaser (Baltimore) because of recurrent severe pain; she had had a delayed transfusion reaction after exchange transfusion and did not wish to be transfused again. Patient H, a 35-year-old man, could not be transfused and had shown no improvement when treated with DDAVP, heparin, or phlebotomies; his mean corpuscular volume (MCV) on admission was 62 but rose to normal after treatment with iron. When his Hickman catheter became infected and had to be removed, he withdrew from the study. None of the patients was physically dependent on narcotics.

RESULTS

Drug Dosage

Drug regimens used and some measures of red cell production and Fb F synthesis are summarized in Table 2. Patients A and C were initially treated with 50 mg/kg 3 days a week, and both became reticulocytopenic³¹; treatment was resumed at lower dosage after recovery from marrow depression, but the HU dosage did not become stable until day 98 for patient A and day 50 for patient C. In patients E and H, F reticulocyte production stabilized after about 3 weeks (Fig 1) and the tabulated data start at that point. Patient G, not shown in Table 2, received only two doses of HU for measurement of its renal clearance and then withdrew from the study. Patient H, followed with some difficulty because of lack of venous access and residence in Georgia, was treated conservatively.

Our earlier studies³¹ indicated that marrow suppression might be related to decreased renal clearance of HU in patients whose kidneys did not function normally.⁴⁴ Serum urea nitrogen and creatinine, creatinine clearance, and HU clearance were measured in each of six patients (Table 1). As previously reported,³¹ the clearance of HU in sickle cell anemia (SS) patients given a standard dose of HU (25 mg/kg) varies considerably. We noted (Table 1) that the F reticulocyte response ratio (absolute F reticulocyte count on day 8 after the clearance study divided by the count before therapy) was inversely correlated with peak and 6-hour HU plasma concentrations, as well as the rapidity of plasma HU clearance as measured by total area under the concentration-time curve (AUC).⁴⁵

Table 1. Creatinine and Hydroxyurea Clearances

Patient	Cr	[HU] _{peak} *	[HU] ₆ *	AUC ₆ †	AUC _∞ ‡	F Retic Response Ratio§
A	0.8	27	6	930	1,070	3.9
C	0.9	71	14	1,700	2,390	1.5
E	0.6	67	22	1,930	3,710	0.4
F	0.4	71	24	1,470	1,470	1.7
G	0.6	36	8	950	1,150	4.7
H	0.9	65	8	1,440	1,650	2.8
r for F retic response ratio	0.14	-0.84	-0.83	-0.97	-0.85	—
P for F retic response	NS	0.034	0.002	0.033	0.040	—

*Concentrations expressed as 10^{-5} mol/L.

†Area under concentration-time curve for 6 hours after test dose.

‡Area under concentration-time curve extended to infinite time.

§Ratio of absolute number of F reticulocytes on day 8 after therapy to pretreatment F reticulocyte number.

Table 2. Red Cell Production and HbF Synthesis

Patient	Days of Rx	HU		Mean HB (g/dL)	Mean MCV (fL)	Mean MCH (pg)	Median MCHC (g/dL)	Mean Abs Retic ($10^3/\mu\text{L}$)	Mean F Retic (%)	Mean Abs F Retic ($10^3/\mu\text{L}$)	Mean F Cells (%)		Max Hb F (%)	Mean Hb F/F Cell (pg)	MCSHC F Cells (g/dL)	Dense Cells (%)
		mg/kg/d	d/wk								F	Hb F				
A	0*†	0	0	11.1	96	34	(36)§	519	4	21	10	1.6	—	5.4	30	—
	0†	0	0	8.9	110	38	33	185	8	15	44	7.9	—	7.2	28	7.3
	98–147	45	2	10.1	121	42	30	249	27	65	39	11.9	17.9	12.8	24	0
	168–262	36	2	11.1	129	45	31	174	28	48	40	12.3	17.9	13.8	24	0.1
	265–378	36	1	8.9	133	46	31	280	26	56	31	8.6	17.4	12.8	25	2.1
	539–685	18	3	10.2	113	39	31	420	20	77	39	8.7	11.7	8.7	27	5.0
C	0*†	0	0	8.0	98	34	(35)§	345	14	48	17	2.9	—	5.8	29	—
	0†	0	0	9.5	122	42	32	243	20	49	62	16.5	—	11.8	25	5.7
	50–867	29	1	9.8	126	41	34	306	31	87	62	18.1	22.9	12.0	23	4.8
E	0†	0	0	8.6	89	31	30	361	26	93	25	2.9	—	3.0	31	0
	19–68	45	3	8.7	104	36	31	277	33	84	48	10.7	16.6	7.1	28	4.2
	69–125	39	3	9.2	111	39	—	252	37	94	57	11.5	16.4	8.0	28	—
F	0	0	0	6.4	91	34	32	450	8	19	11	1.1	—	3.4	34	29.0
	20–27	42	2	6.4	102	38	34	374	24	78	21	3.1	3.1	5.6	33	0
	28–44	42	1	6.5	103	37	34	389	19	39	—	4.6	5.2	5.2	31	0
H	0	0	0	8.6	64	21	29	215	3	4	6	1.0	—	3.5	27	0
	33–86	14	3	9.9	84	27	29	270	17	44	25	3.1	4.7	3.3	28	1.0
	90–110	20	3	10.2	92	30	29	339	18	63	37	5.3	5.6	4.3	28	0
	195–239	7	7	10.7	89	30	32	329	11	35	35	7.9	9.6	6.8	26	7.0

F cell characteristics were calculated as follows: MCH in S Cells = MCH in F Cells; F/F Cell = [(MCH) (%F)/% F Cell]; S/F Cell = MCH - F/F Cell; S/S Cell = MCH; MCSHC in F Cells = (S/F Cell/MCV).

*Before AzaC; transfused.

†Two weeks after end of AzaC therapy.

‡Transfused.

§Coulter counter; other values are based on density gradient centrifugation.

Hematologic Effects

The proportion of F reticulocytes, F cells, and Hb F in hemolysates rose during therapy with HU (Table 2). Indices of Hb F production varied considerably over time but appeared to behave in a more concerted fashion in patient A than in patient C. Patient A has shown an increase in Hb F

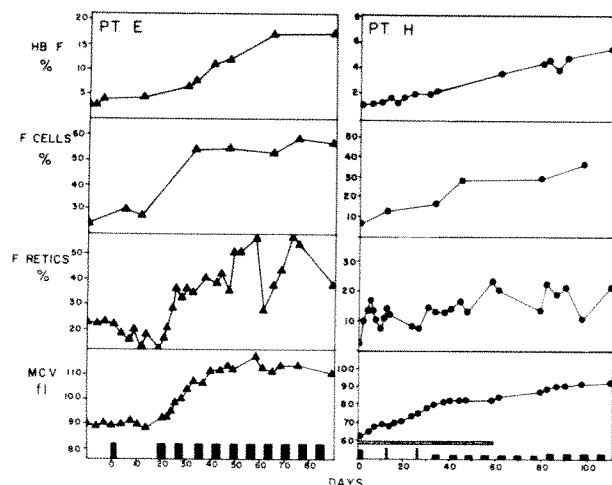


Fig 1. Response of patients E and H to HU. E had been transfused, and H was iron deficient before therapy. Black bars represent HU and the stippled bar FeSO₄ treatment.

production in recent months, coincident with a change in treatment from three to four days per week. The change has been gradual, over a period of 5 months. Patient E reached maximum F cell and Hb F levels after about 2 months of treatment; patient H, treated more conservatively, showed increasing levels when therapy was stopped after 3½ months (Fig 1). Absolute reticulocyte counts fell below baseline at some points in most patients but remained over 200,000/mm³ (except during folate deficiency in patient A). No patients developed thrombocytopenia or severe neutropenia (<2,000/mm³), but patient C exhibited intermittent lymphocytosis (see below under Clinical Responses). There was no clear relationship between DNA polymorphisms within the γ - δ - β gene cluster^{42,46,47} and F cell response.

MCV and MCH rose markedly in patients who responded (Table 2), but there was little or no change in median MCHC as estimated from phthalate ester density gradients. As expected,⁴⁸ the calculated Hb F/F cell and number of F cells varied independently, and the concentration of Hb S within F cells decreased. The early rise in Hb F (and MCV) observed in patient H may have been related to treatment of iron deficiency (Fig 1).^{49,50}

Clinical Responses

Patient A's crises have become shorter and milder (Fig 2) but still persist. Patient C has occasional attacks of a few hours' duration but has resumed full-time employment.

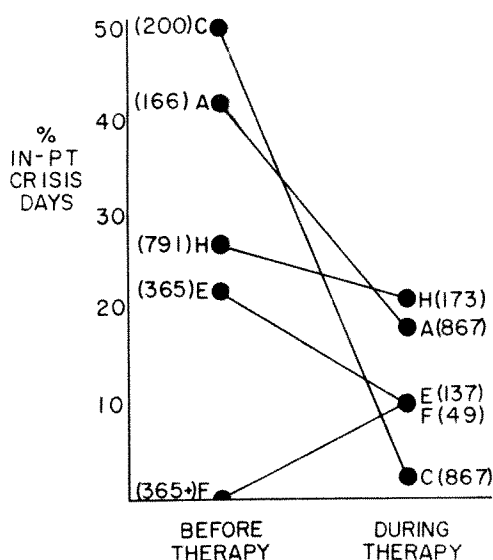


Fig 2. Clinical responses to HU therapy. Days of observation are shown in parentheses, and percentages of those days spent in hospital because of vaso-occlusive crises are plotted.

Patient E continued to complain of intermittent abdominal pain during treatment; a biliary stricture was discovered, and reconstructive surgery was done. Her complaints disappeared, and she elected to discontinue therapy. Patient F showed no healing of her ankle ulcer and only a small response to HU therapy. The crisis she experienced while hospitalized was quite mild but represented a change from her usual condition. Patient H is still incapacitated by his disease but did not receive enough HU to be considered a treatment failure.

An attempt was made to relate the occurrence of inpatient crises in patients A and C to one or more of the measurements made on red cells. There was little difference between sets of data collected during vaso-occlusive crises, between crises, and during the week preceding a crisis (while the patient was pain free). In particular, there were no differences in red cell distribution width (RDW) or the proportion of dense cells between the three clinical situations. It should be noted that patient C had almost as many crises as patient A, but they lasted for one to two days, rather than for weeks at a time.

Untoward Effects

Toxicity. Patient A developed megaloblastic anemia on day 416, which responded promptly to folic acid; he had run out of tablets several months before. On day 576 he was admitted with atrial fibrillation, which responded to a brief course of digoxin and has not recurred; the etiology was thought to be cardiac hemochromatosis. An area of altered density in his liver on abdominal sonography could not be detected by computed tomography (CT) scan and was thought probably to be due to fibrosis.

Patient C, who has intermittent lymphocytosis, developed disseminated herpes zoster on day 335. Response to acyclovir was prompt; his varicella zoster antibody titer was 1:200 and rose to >1:2,000; he was not anergic; proportions of T and B

cells were 60% and 30%; and his T4-T8 ratio was 1:1. At the beginning of AzaC therapy his differential white count was normal, but he had too few T cells to count; the T4-T8 ratio was 0.5 when he was switched to treatment with HU. It is currently 0.6 and that of patient A is 1.2.

Patients F and H complained of mild nausea after taking several capsules of HU simultaneously, but these complaints diminished when the dose was divided or lowered. Patient G developed a high fever after one HU clearance study but did not after a second; her participation in the study was terminated by mutual agreement.

Chromosomal analyses. During therapy with AzaC patients A and C were found to have an increased number of chromosomal structural rearrangements or deletions; unfortunately, chromosomal studies were not carried out before AzaC therapy. Specimens from patients E, G, and H yielded abnormal patterns before treatment with HU. Subsequent analyses during HU therapy (Fig 3) showed no progression of changes in patients A, C, or H. Many affected cells in patient G had more than one of the acquired aberration, suggestive of a recent viral infection.⁵¹ She also was the only patient transfused within several weeks of chromosomal analysis. Potential causes of the observed rearrangement and breaks included an effect of prior transfusion, narcotics, street drugs, unrecognized viral infection, or a complication of the therapeutic regimen.⁵²

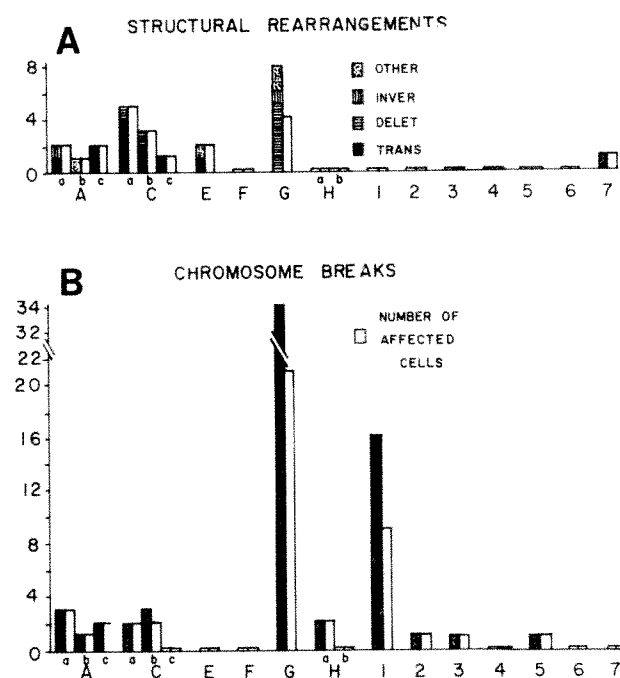


Fig 3. (A) Chromosomal abnormalities in patients treated with HU (A through H) and in untreated SS controls studied at the same time (1 through 7). Subscripts a through c represent serial samples: At least 30 cells were examined in each analysis (mean = 61). (B) Solid bars represent numbers of breaks observed, and open bars represent numbers of affected cells. The control patients were 6 to 34 years of age (mean = 24); five of them had been extensively transfused, but only patient 1 had received significant amounts of narcotics. INVER = inversion; DELET = deletion; TRANS = translocation.

Two groups of controls were studied at the same time as the patients who were treated with HU. Seven sickle cell anemia patients, most of whom had been extensively transfused but had little exposure to narcotics, served as untreated controls and were studied at the same time as the treated patients. One SS control had a single chromosomal rearrangement as did two of 13 AA controls. One SS control had a number of chromosomal aberrations, but as noted in the specimen from patient G, there was more than one aberration per affected metaphase (17 aberrations/9 affected metaphases), which is compatible with a recent viral infection.⁵¹

No structural rearrangement or deletion was found in more than one cell, and the chromosomal breaks were random. Abnormalities were found in two of 13 normal (AA) controls, but three cells were involved in each of them.

Carcinogenesis. No patient has developed evidence of a malignant disorder on physical examination. Chest x-rays, bone surveys, abdominopelvic CT scans, and sonograms of the testicles have been done in patients A and C at 6-month intervals; neither they nor physical examinations have disclosed any new abnormalities since the beginning of experimental therapy with AzaC (44 months in patient A and 35 months in patient C).

DISCUSSION

Prediction of Response

We have recognized three of the factors that appear to determine the response of SS patients to HU: HU clearances, baseline F reticulocyte production, and dosage regimen.

Earlier we observed that F reticulocyte production after HU administration was related inversely to the serum creatinine level and directly to the rapidity of HU clearance from plasma.³¹ Those suggestions are supported by the data reported here. In the previous report, three patients with serum creatinine above 1.0 mg/dL showed marrow suppression and failure to increase their F reticulocyte levels. No patient in this report has a creatinine level above 0.9, and all responded with substantial increases in F cell production. In the earlier report, rapid HU clearance appeared to be associated with a high F reticulocyte response. Here studies of HU clearance (including AUC analyses as well as plasma levels) could be used to predict initial and highest mean F reticulocyte response ratios. They could not be used to predict highest mean F cells, Hb F, or F/F cell, nor did they predict the mean F reticulocyte count for a given dose of HU per se. For example, the patients with the fastest (A) and slowest (E) clearances had the highest and lowest F reticulocyte response ratios, but the maximum mean F cell and Hb F levels they achieved were similar (Tables 1 and 2).

A second factor involved in determining the response to HU is the patient's genetically determined baseline F reticulocyte production level.^{53,54} This level ranges from 4% to 50% F reticulocytes,^{48,53} is inherited,⁵⁴ and is fixed some time after the first year of life.⁵⁵ As we suggested earlier, the patients with high initial counts (C and E) eventually achieved the highest mean F reticulocyte counts during treatment, and C achieved the highest F cell count, Hb F level, and Hb F/F

cell (Table 2). DeSimone obtained similar results in baboons treated with AzaC, in a situation where inherited variations of F cell production are similar to those in humans.²⁴ DNA polymorphisms around the γ - δ - β gene cluster have been reported to correlate with increased levels of Hb F,^{46,47,56} but such analyses in our patients did not yield a convincing Hb F pattern.

A third determinant of the F reticulocyte response is dosage regimen. The two patients studied for several years appear to have different optimal regimens. During recent months patient A responded better to HU when it was given four days a week rather than three, and Alter and Gilbert have reported that patients receiving HU for myeloproliferative syndromes produce more Hb F on a daily rather than an intermittent dosage schedule.⁵⁷ Our present hypothesis is that patients' ability to rapidly excrete HU and their baseline F reticulocyte production levels are important determinants of their response to HU, but that the influence of the former, at least, can be modified by manipulation of dosage regimens. Individuals with low baseline F reticulocyte levels and delayed HU clearance will be the least likely to respond to therapy, and vice versa. Patients who fall between these extremes can be expected to show some response, but the magnitude of that response is as yet undetermined.

Hematologic Effects

The best dosage regimen should be one that maximally increases both the number of F cells and the amount of Hb F within those cells. To assess production of F cells, we have measured absolute numbers of F reticulocytes, which reflect changes in erythropoiesis as well as Hb F synthesis. Uncertainties about relations between dosage regimen and response have prompted us to continue direct measurements of F cell production. Macrocytosis was a constant result of treatment, damped in patient H by iron deficiency and coexisting α -thalassemia (α -/ α); PF Milner, personal communication, December 1985). As was observed with AzaC, MCH rose proportionately, the increase reflecting an increase in the amount of Hb S/per cell as well as Hb F in the F cells. We have previously reported that the proportion of dense cells (MCHC > 37 g/dL) did not decrease when patients A and C were treated with AzaC.²⁰ Here we show that the proportion of such cells did not decrease during HU therapy (Table 2), nor did it change in relation to crises.

The intraerythrocytic concentration of Hb S, a prime determinant of sickling tendency,⁵⁸ fell in F cells. In patient C the mean corpuscular sickle hemoglobin concentration (MCSHC) (Table 2) fell from about 32 to 22 g/dL in F cells. This fall in MCSHC would prolong the delay time (tenth time)^{59,60} of his F cells from 30 minutes to three to four minutes, or decrease their polymer fraction at 70% oxygen saturation⁵⁸ from 0.12 to 0.

Clinical Responses

In patients A and C, who did not know their F cell or Hb F levels, there was a relation between admissions for painful crises and symptom-free periods and their F cell production, strongly suggesting that the improvement we observed was not a placebo effect. Crises were less severe and less frequent

when Hb F was 8% to 10% and there were 55% to 60% F cells; they were virtually eliminated when Hb F was 15% to 18% and there were 60% to 70% F cells. The latter levels are lower than the >20% Hb F reported by Powars et al⁶¹ to be associated with fewer painful events and lower than the mean value of 25% found in mildly affected Saudi Arabian SS patients.^{11,12} On the other hand, patient E epitomizes the hazard of interpreting subjective complaints: she did not improve clinically despite a substantial increase in Hb F synthesis and then improved so rapidly after biliary tract reconstruction that she considered HU therapy unnecessary.

Untoward Effects

Toxicity. Atrial fibrillation in patient A was more likely related to hemochromatosis than to HU therapy. Development of mild but disseminated herpes zoster in patient C may have been related to HU therapy, but the patient's excellent clinical response to HU has encouraged him (and us) to continue treatment. Abnormal numbers of lymphocytes and striking abnormalities among his T cells may be related to prior transfusions^{62,63}; these changes have not progressed during treatment, nor have similar but less marked changes in patient A. Bone marrow depression has not been a problem with the doses we used, after the effect of subclinical renal disease was appreciated.

Chromosomal analyses. Discovery of chromosomal breaks and rearrangements during AzaC therapy in patients A and C prompted us to carry out chromosomal analyses in all other patients, as well as in untreated SS patients and normal controls. Some patients who have not received any cytotoxic drug have chromosomal abnormalities (Fig 3), and serial studies of patients A, C, and H showed that such

abnormalities have not progressed during treatment. Hydroxyurea is reported to be a strong inducer of chromosomal breaks (a "clastogen") in vitro but to have weak activity in inducing sister-chromatid exchanges.⁶⁴ Lymphocytes from patients with psoriasis treated with HU yielded abnormal karyotypes, but no control studies in untreated psoriatics were performed.⁶⁵ Cytogenetic aberrations do occur in normal volunteers⁶⁶; our data must be interpreted cautiously because our patients have been exposed to a variety of substances and treatments in addition to HU. Studies of patients and normal controls support such an interpretation, but it would seem prudent that patients receiving HU avoid conception or pregnancy.

Carcinogenesis. The risk of carcinogenesis in humans from HU is unknown. The drug is teratogenic and mutagenic^{33,34} in laboratory animals, but aspirin was equally teratogenic in a recent study.⁶⁷ HU induces sperm abnormalities in mice⁶⁸ but suppresses two-stage carcinogenesis in mouse skin^{69,70} and was considered noncarcinogenic in two small studies in animals.^{32,71} The best clinical evidence is inconclusive: patients with polycythemia vera who have been treated with HU show insignificant differences in the incidence of neoplasms when compared with patients treated only by phlebotomy.⁷² The series is not large (53 patients), and observations have continued for a median of only 5 years, but we have interpreted these data to our patients as indicating "safety unproved, but risk probably small."

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Lithium Augments GM-CSA Generation in Canine Cyclic Hematopoiesis

By William P. Hammond, Elin R. Rodger, and David C. Dale

Cyclic hematopoiesis in gray collie dogs can be cured by lithium treatment. We examined the mechanism of lithium's effect by developing an assay for the canine equivalent of GM-CSF (called GM-CSA). Phytohemagglutinin (PHA)-stimulated canine blood mononuclear cells produce GM-CSA in a dose-dependent manner; this GM-CSA stimulates more neutrophil-containing colonies than does endotoxin-treated dog serum. Production of GM-CSA by PHA-stimulated normal dog cells was not altered by lithium.

STUDIES OF CYCLIC HEMATOPOIESIS in gray collie dogs have shown that the regular 12-day periodicity of cell count fluctuations is due to changing marrow production rates and can be transferred or cured by bone marrow transplantation.¹⁻⁶ We and others have shown that the numbers of committed erythroid and granulocytic progenitor cells fluctuate over the cycle period and do so out of phase from their differentiated progeny.⁷⁻¹⁰ In studies of the effects of endotoxin and lithium treatment on these dogs, we showed that treatment abrogated the cyclic fluctuations of the progenitors as well as the blood cell counts.^{7,10-12} We also showed no differences in serum colony-stimulating activity (CSA) levels, responsiveness to CSA, or tritiated thymidine suicide rates of the granulocyte-macrophage progenitor cells (CFU-c) over the period of the cycle and were unable to show that successful treatment induced any change in these variables.^{7,12}

Because the assay system we had used to measure CSA produced colonies from the CFU-c that were primarily macrophagic (with a minor population of neutrophilic progenitor cells), we considered the possibility that an alternative source of CSA containing a canine equivalent of GM-CSF would be a more useful probe into the genetically aberrant regulatory system. Phytohemagglutinin (PHA)-stimulated blood mononuclear cells have been shown to elaborate GM-CSF in human and murine systems and served as the model system for these studies.¹³⁻¹⁶ We have now demonstrated a canine equivalent of GM-CSF (which we here call GM-CSA), have examined its elaboration by normal and gray collie dog mononuclear cells, and have measured lithium's effect on its generation.

MATERIALS AND METHODS

Dogs. Male and female gray collie dogs from 2 to 60 months of age and weighing from 5 to 25 kg were housed in individual cages or runs as previously described.^{7,10-12} Normal mongrel dogs and normal littermates of the gray collies served as controls. Blood specimens were routinely drawn from the cephalic vein of unanesthetized dogs; blood lithium levels were drawn before that day's dose when indicated. Dogs treated with lithium were given lithium carbonate capsules, 300 mg daily, and dosages were adjusted to keep serum lithium levels between 0.5 and 1.4 mEq/L as previously described.^{11,12}

Blood counts and bone marrow aspirates. Blood counts were performed by standard methods. For marrow aspirates the dogs were anesthetized with intravenous thiamylal sodium (Bio-tal; Bio-Ceutic Laboratories, St Joseph, MO), 15 to 20 mg/kg. Bone marrow was obtained from the humerus, femur, or iliac crest, heparinized, and diluted in 5 mL tissue culture medium.

However, cells from gray collies during their neutrophilic period increased their GM-CSA when lithium (2 mEq/L) was added to low doses of PHA, whereas neutropenic gray collie cells did not. These data suggest that lithium could modulate cyclic hematopoiesis by increasing intramedullary GM-CSA at the time when marrow neutrophilic progenitor cells are at their nadir.

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In vitro bone marrow culture. Granulocyte-macrophage progenitor cells were grown as previously described.^{7,12} Final plating suspensions contained 1 to 2×10^5 nucleated cells per milliliter, 10% fetal calf serum, 0.3% agar, and a source of CSA. All assays were run in triplicate. Incubations were carried out for variable numbers of days, as indicated in Results. Colonies of 50 or more cells were counted with an inverted microscope and results expressed as colonies per plate or 10^5 nucleated marrow cells (total CFU-c) or as a percentage of control colony growth. Colony subtypes were identified as neutrophil, macrophage, or mixed colonies as described previously.¹²

Colony-stimulating activity. The standard CSA used in our earlier studies was postendotoxin dog serum (PEDS).^{7,12} Such serum is maximally active when added at 5% to 10% vol/vol in dog marrow cultures and was used as the control CSA source in the current studies.

PHA-stimulated leukocyte conditioned medium (PHA-LCM) was prepared in a manner similar to that used for human and murine cells.¹³⁻¹⁵ Dog peripheral blood was heparinized (beef lung heparin; Upjohn Co, Kalamazoo, MI, 20 U/mL), diluted 1:1 in Hanks' buffered saline solution (BSS; GIBCO, Grand Island, NY) and layered over Ficoll-Hypaque solution (density 1.074 to 1.076, osmolality 295 to 300). After spinning at 400 g for 35 minutes at 4 °C, the mononuclear cells were removed from the interface zone, washed three times, resuspended in fresh medium, and counted. Differential count of mononuclear cells from neutropenic gray collies revealed fewer than 0.2% neutrophils, $47\% \pm 5\%$ lymphocytes, and $44\% \pm 5\%$ monocytes. Both normal dogs' and neutrophilic gray collies' mononuclear cell populations contained more neutrophils (from 7% to 15%, $P < .01$) and fewer monocytes (from 20% to 28%, $P < .05$) than neutropenic gray collies but had similar numbers of lymphocytes ($P > .10$). Differential counts of neutrophils, monocytes, and lymphocytes were not statistically significantly different between normals and neutrophilic gray collies ($P > .08$). The cells were placed in plastic tissue culture flasks (T-75; Falcon, Cockeysville, MD) containing supplemented tissue culture medium, 20% fetal calf serum (GIBCO), PHA (M form) (GIBCO) at final dilutions of 1 to 50 μ g/mL, and in some flasks, lithium chloride at 2×10^{-3} mol/L. These flasks were incubated at 38 °C, in 5% CO₂ for seven days. The supernatants were then removed, centrifuged twice at 1,000 g for 15

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minutes to remove cellular debris, and stored at -10°C until assayed for CSA. PHA-LCMs were assayed at 10% vol/vol with 5% normal dog serum to compare with 5% PEDS.

Modulation of CSA. Modulation of CSA in these PHA-LCMs was tested in two ways. To determine whether LiCl modulated CSA generation by mononuclear cells independent of T lymphocytes, some flasks were prepared with addition of the potent T lymphocyte inhibitor cyclosporine at $3\text{ }\mu\text{g/mL}$ (kindly provided by Dr Joachim Deeg, Fred Hutchinson Cancer Research Center, Seattle). In addition, to eliminate the contribution of carryover of lithium in PHA-LCMs prepared with LiCl, we dialyzed two sets of conditioned media against three changes of Hanks' BSS with $100 \times$ volume excess at 4°C . Dialyzed conditioned media were then compared with each other and to nondialyzed aliquots of the original PHA-LCM.

Tritiated thymidine ($^3\text{H-TdR}$) suicide and velocity sedimentation studies. We estimated cell cycle status of CFU-c by $^3\text{H-TdR}$ suicide as previously described.⁷ The percentage of thymidine suicide was calculated by subtracting $^3\text{H-TdR}$ -treated CFU-c growth from similarly treated cells not exposed to $^3\text{H-TdR}$ ("control") and dividing by "control" growth. Controls demonstrated that nonspecific toxicity was not involved. To calculate CFU-c suicide with different sources of CSA, the same treated and control cell suspensions were stimulated with PEDS and with PHA-LCM.

Velocity sedimentation at unit gravity was performed by previously published methods.^{17,18} Marrow mononuclear cell suspensions were loaded into the Sta-Put chamber (150×10^6 cells in 50 mL), followed by a linear gradient of 15% to 30% fetal calf serum in tissue culture medium. After $3\frac{1}{2}$ hours of sedimentation, 50-mL fractions were collected, cell counts and smears obtained, and assays for CFU-c performed using either PEDS or PHA-LCM for CSA.

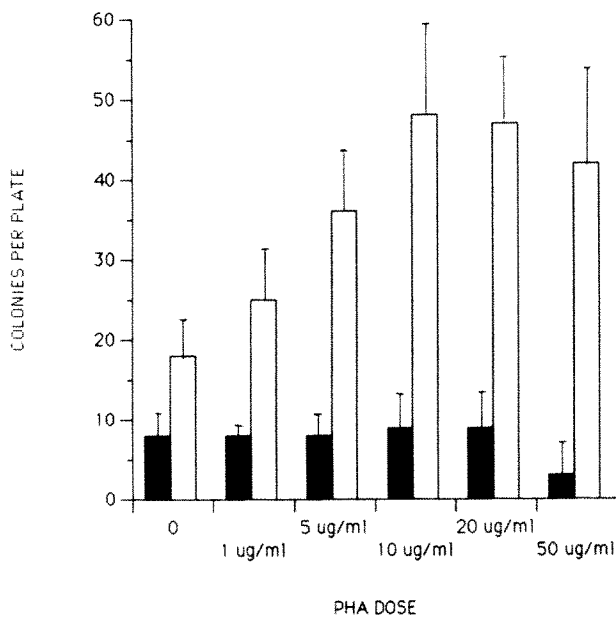


Fig 1. Total CFU-c number when stimulated by 5% normal dog serum plus either PHA-LCM (□) or PHA added directly to dog marrow cells (■). The concentration of PHA used to stimulate blood mononuclear cells to make LCM varied from 1 to $50\text{ }\mu\text{g/mL}$ and was compared with the effect of adding the same PHA concentration directly to marrow cells. Results are expressed as mean $\pm$ SEM colony numbers per plate containing 1×10^6 nucleated bone marrow cells and assayed at days 8 through 10 (N = four experiments, each experimental point representing a mean of triplicate plates).

RESULTS

Colony stimulating activity in PHA-LCM. Initial experiments confirmed that PHA-LCM made from dog peripheral blood mononuclear cells incubated for seven days do stimulate the growth of colonies from normal dog bone marrow growing at 10 days of incubation. This is a dose-dependent stimulation with a broad plateau of activity evident from 10 to $50\text{ }\mu\text{g/mL}$ that is not due to carryover of PHA into the marrow, since direct addition of PHA to marrow cells is not stimulatory (Fig 1).

Colonies formed with PHA-LCM. Using one lot of PHA-LCM made from normal dogs with PHA at a concentration of $50\text{ }\mu\text{g/mL}$, we next examined the time course of development of macrophage and neutrophilic colonies from normal dog marrow cells. As previously shown, the colonies growing early in culture were largely macrophage colonies, whereas those growing later were a mixture of neutrophilic and macrophage colonies. Plotting colonies per plate (10^5 marrow cells), PEDS and PHA-LCM showed distinctly

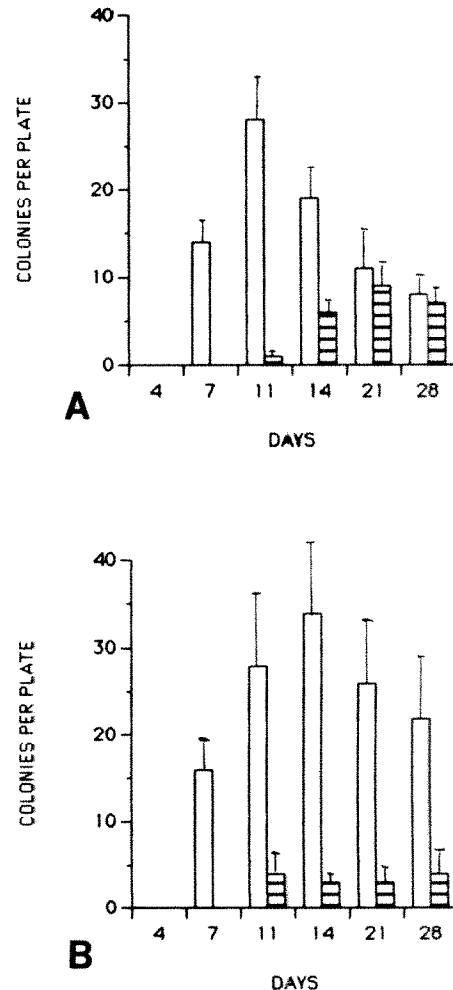


Fig 2. The effect of (A) PHA-LCM and PEDS (B) on morphological subtypes of colonies at various days in culture. □, Macrophage colonies; ▨, neutrophil-containing colonies (pure neutrophil and mixed neutrophil-macrophage colonies). Results expressed as in Fig 1 (N = five experiments).

different patterns of growth, with a late-occurring burst of neutrophil-containing colonies (neutrophil and mixed) when PHA-LCM was used as the source of CSA (Fig 2).

We then explored the subtypes of colonies formed using PEDS and PHA-LCM as stimulators for gray collie dog marrow target cells and compared them with results with normal dog marrow cells. With normal dog marrow cells, PEDS stimulated a peak number of CFU-c by day 10 or 11, which then remained elevated to days 21 and 28, while PHA-LCM caused a similar rise in total colony number. Subtypes of CFU-c were then determined at day 10 and at day 16 or 17 and marked differences between PEDS and PHA-LCM were noted (Table 1). At day 10 only 11% of colonies were neutrophilic with PEDS as stimulator, whereas 26% were neutrophilic with PHA-LCM ($P < .05$). This difference was further magnified at days 16 to 17, when 2% were neutrophilic with PEDS and 59% were neutrophilic with PHA-LCM ($P < .001$) (Table 1). In general, the same features were evident using gray collie marrow cells as targets: PEDS stimulated mostly macrophagic colonies, whereas PHA-LCM stimulated more neutrophilic colonies, especially at days 16 and 17 (Table 1).

Colony-forming cell characterization. To determine whether PEDS and PHA-LCM stimulated different colony-forming cells, we layered dog bone marrow mononuclear cells over a velocity sedimentation gradient. Assays of each fraction for CFU-c stimulated by either of the stimulators demonstrated that the cell populations forming colonies had the same general patterns of sedimentation with modal sedimentation velocities of 7.1 mm/h (Fig 3).

Tritiated thymidine suicide studies to estimate the cell cycle characteristics of normal dog marrow cells stimulated by PEDS and PHA-LCM showed that CFU-c grown with PEDS had a suicide rate of $45\% \pm 7\%$ (comparable to our prior report⁷), whereas those grown with PHA-LCM had a lower suicide rate of $29\% \pm 6\%$ ($n = 4$, $P < .05$, paired t test).

Effect of lithium and PHA on cyclic neutropenic blood mononuclear cells. Initial trials showed that addition of lithium *in vitro* to peripheral blood mononuclear cells by itself did not enhance the elaboration of CSA (GM-CSA)

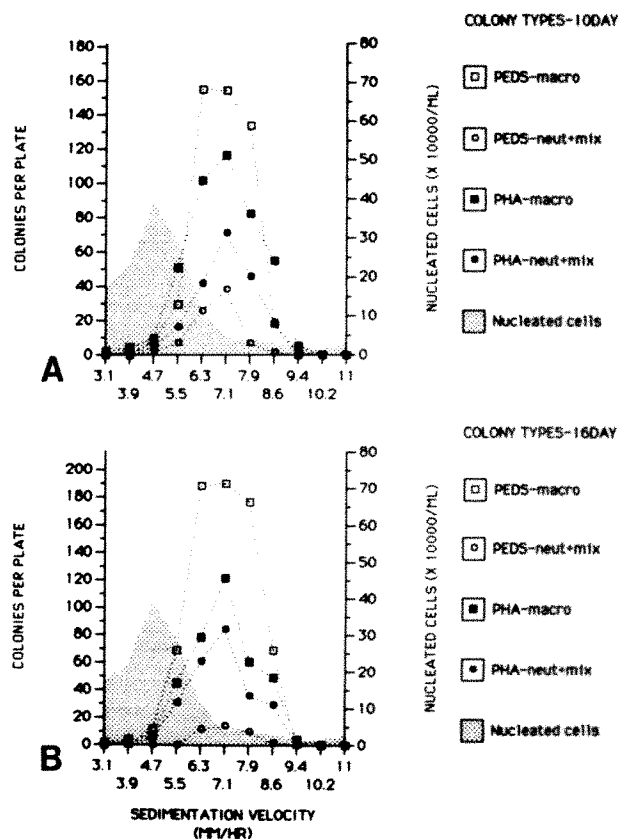


Fig 3. Velocity sedimentation profiles of dog CFU-c assayed at day 10 (A) and day 16 (B). Colonies formed when PHA-LCM was used as a stimulator are shown by solid symbols; PEDS, by open symbols. Modal sedimentation velocity for all CFU-c was 7.1 mm/h, while the modal sedimentation velocity for all low-density marrow cells was 4.7 mm/h (shaded area).

(data not shown). We proceeded to examine the effects of adding lithium to mononuclear cells stimulated by PHA to produce GM-CSA. Lithium at 2 mmol/L (2 mEq/L) caused no increase in CSA generation at plateau concentrations of PHA (eg, 50 $\mu\text{g/mL}$), but at submaximally stimulatory concentrations (1 and 5 $\mu\text{g/mL}$) it markedly enhanced the

Table 1. Colony Subtypes Stimulated by PEDS and PHA-LCM

Target Marrow	Stimulator	Day 10		Days 16-17	
		Neutrophils (%)	Macrophages (%)	Neutrophils (%)	Macrophages (%)
Normal	PEDS, 10%*	11 $\pm$ 4	89 $\pm$ 4	2 $\pm$ 1	96 $\pm$ 1
	PHA-LCM, 10%†	26 $\pm$ 5	67 $\pm$ 17	59 $\pm$ 3‡	29 $\pm$ 4
Gray collie	PEDS, 10%	9 $\pm$ 4	91 $\pm$ 4	15 $\pm$ 5	85 $\pm$ 5
	PHA-LCM, 10%	14 $\pm$ 4	86 $\pm$ 4	69 $\pm$ 5‡	29 $\pm$ 5

Plates were scored for percentage of subtype from each of three to 11 dog marrows; mean percentage $\pm$ SEM, n = total number of marrows assayed.

*10% vol/vol.

†PHA-LCM—phytohemagglutinin-stimulated (50 $\mu\text{g/mL}$) leukocyte conditioned medium from a single normal dog, 10% volume/volume.

‡Significantly different ($P < .05$) from colony subtypes stimulated by PEDS.

production of GM-CSA by mononuclear cells obtained from gray collies during neutrophilia (cycle days 8 through 12) (Fig 4). Neither normal dogs nor gray collies during neutropenia (cycle days 2 through 4) showed this stimulatory effect. Gray collie dogs whose cycling had been eliminated by lithium treatment in vivo likewise failed to show this enhancement by in vitro addition of lithium (Fig 5).

Inhibitor assays. Experiments were performed to determine whether addition of Li to gray collie mononuclear cells caused a reduction of an inhibitor present in the untreated (no Li) LCMs or an increase in a stimulator. One-to-one mixtures of control PHA-LCM (made by normal dog mononuclear cells stimulated with 50 $\mu\text{g}/\text{mL}$ PHA) and freshly prepared gray collie LCMs were performed. When a total of 10% LCM (5% control PHA-LCM plus 5% test PHA-LCM) was added to normal dog marrow cell targets, we found no decrease in growth compared with that expected with control PHA-LCM alone (Table 2) ($P > .30$). Parallel assays using normal dog PHA-LCMs similarly showed no evidence for inhibitors (data not shown).

Dialysis and blocking experiments. Additional studies of these conditioned media were carried out to determine that lithium carryover into the target marrow cells was not responsible for the stimulatory effect. In dialyzed conditioned media there was a slight decrease in CSA, but the same differences in stimulatory activity between lithium-treated and lithium-untreated conditioned media were readily demonstrated (Table 3). Thus, whereas at PHA concentrations of 50 $\mu\text{g}/\text{mL}$, Li had no effect on CSA in either dialyzed or undialyzed LCMs, at the lower concentrations of PHA, both dialyzed and undialyzed LCMs showed significant increases in CSA when Li was added. In addition, studies were performed in which cyclosporine was added to the mononuclear cell suspensions that were incubated to

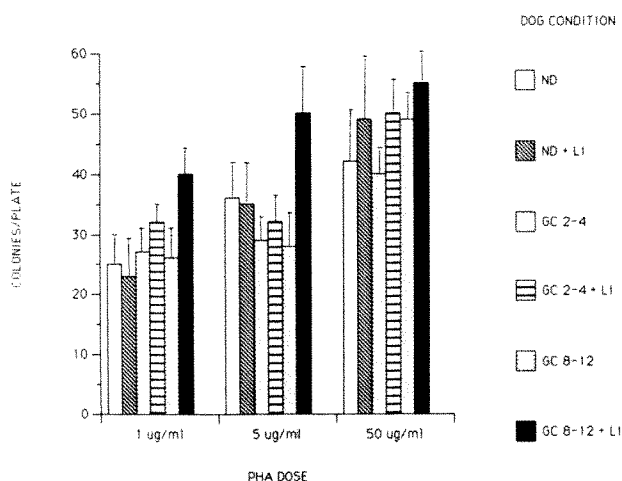


Fig 4. Effect of lithium (2 mmol/L) on PHA-induced dog GM-CSA. PHA-LCMs were made using PHA at 1, 5, and 50 $\mu\text{g}/\text{mL}$ from normal dogs (ND) and from gray collies during neutropenia (GC 2 through 4) or during neutrophilia (GC 8 through 12), either without or with lithium (+Li). PHA-LCMs were all assayed against normal dog marrow cells and results are reported as in Fig 1 (N = seven experiments). Neutrophilic gray collie cells (GC 8 through 12) showed a significant ($P < .05$) increase when Li was added at 1 and 5 μg PHA.

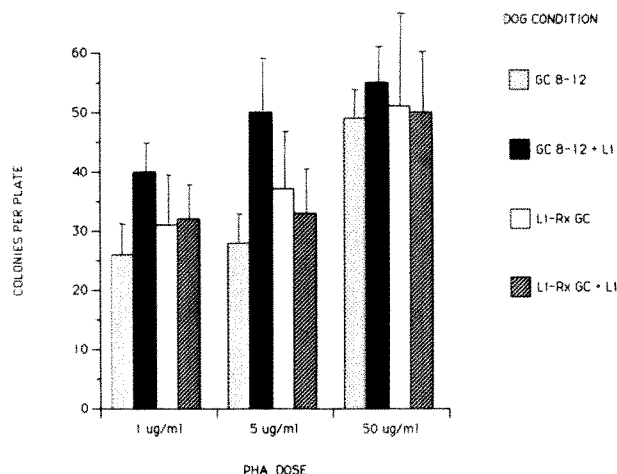


Fig 5. Effect of lithium treatment in vivo on lithium responsiveness of PHA-induced dog GM-CSA. Untreated gray collie cells (GC 8 through 12) show significant ($P < .05$) increase with addition of Li at 1 and 5 μg PHA, whereas lithium-treated gray collie cells (Li-Rx GC) showed no change with addition of Li in vitro. Results presented as in Fig 1 (N = four experiments).

produce CSA. When cyclosporine was added at 3 $\mu\text{g}/\text{mL}$ in the presence or absence of lithium, there was no detectable CSA in the supernatants (Table 4). Addition of cyclosporine directly to the bone marrow assay system demonstrated no inhibitory effects (data not shown).

DISCUSSION

Previous studies of the pathogenesis of cyclic hematopoiesis in gray collie dogs have clearly shown the defect to be one of fluctuating levels of marrow cell production and have documented marked swings in the numbers of committed granulocytic progenitor cells in blood and marrow.¹⁻¹⁰ Although several studies have examined levels of putative

Table 2. Assay for Inhibitors in PHA-LCMs

	Colonies/ 10^5 Cells (n = 4)
Controls	
Without additions	24 $\pm$ 10
10% PHA-LCM (control)	53 $\pm$ 11
Test LCMs	
10% PHA-LCM—1 $\mu\text{g}/\text{mL}$	40 $\pm$ 12
10% PHA-LCM—5 $\mu\text{g}/\text{mL}$	40 $\pm$ 10
10% PHA-LCM—1 $\mu\text{g}/\text{mL}$ + Li	38 $\pm$ 13
10% PHA-LCM—5 $\mu\text{g}/\text{mL}$ + Li	41 $\pm$ 13
Mixtures (1:1, total = 10% vol/vol)	
Control + Test 1	66 $\pm$ 15
Control + Test 2	56 $\pm$ 12
Control + Test 3	55 $\pm$ 14
Control + Test 4	54 $\pm$ 14

Controls refer to cultures containing 5% normal dog serum. Test LCMs were conditioned media made from neutrophilic gray collies with 1 or 5 $\mu\text{g}/\text{mL}$ PHA with or without lithium (Li). Control refers to control above made with normal dog cells and PHA at 50 $\mu\text{g}/\text{mL}$. Two sets of freshly prepared GC PHA-LCMs were prepared and assayed a total of four times against normal dog marrow cells.

Table 3. Dialysis of Gray Collie PHA-LCM

PHA Concentration	Colonies per 10 ⁵ Nucleated Normal Marrow Cells	
	Undialyzed	Dialyzed
No Li added		
1 µg/mL	23 ± 4	22 ± 13
5 µg/mL	17 ± 3	22 ± 4
50 µg/mL	44 ± 8	30 ± 9
Li added (2 mol/L)		
1 µg/mL	40 ± 9*	31 ± 9†
5 µg/mL	C	28 ± 10
50 µg/mL	43 ± 18	34 ± 13

PHA-LCM was from "neutrophilic" gray collie cells (cycle days 8 through 12). Colony numbers are means of duplicate assays of the same dialyzed and undialyzed LCMs, representing six plates for each number, ± one SD. C, specimen contaminated during cultures.

*Significantly different from CSA of LCM made in the absence of lithium ($P < .001$, paired t test).

†Significantly different from CSA of LCM made without lithium ($P < .05$, paired t test) and from CSA of undialyzed LCM ($P < .05$).

granulocytic regulators in this disease,^{7,19,20} each has had significant drawbacks. In the earliest report, dog urine was assayed for activity stimulating murine cell proliferation and questions were raised about species specificity as well as the possibility that the "CSA" was the result of endotoxemia during neutropenia rather than being the cause of the cyclic cell production.¹⁹ In the other two reports, assays used dog marrow cell targets but did not distinguish between neutrophilic and macrophagic "CSA." In our studies the CSA being measured appeared to be the result rather than the cause of neutropenia,⁷ a finding concordant with that of Greenberg et al in the analogous human disorder.²¹ In addition, we found that serum levels of CSA were not altered by either endotoxin or lithium treatments, two therapies that

eliminate cyclic neutropenia in these dogs.^{7,12} However, the lack of neutrophilic specificity of the CSA assays that had been published suggested that a new assay was needed and led directly to the present studies of GM-CSA.

These data show that PHA stimulates dog blood mononuclear cells to produce a CSA with neutrophilic activity. This activity has characteristics similar to those reported for GM-CSF in mice and humans in that it stimulates both G and M colonies, it affects colony-forming cells that grow somewhat later in the cultures, and it appears to stimulate a different subpopulation of CFU-c than does the PEDS as defined by different ³H-TdR suicide rates.¹⁵ Because PHA stimulation is well known to stimulate lymphocyte production of GM-CSF and has been used to purify its cDNA,¹⁶ these results were not unexpected. The ability of cyclosporine to block the elaboration of this material is consistent with its ability to inhibit T lymphocyte function and block production of megakaryocytic CSA.²²⁻²⁴

Knowing that this GM-CSF-like activity (GM-CSA) was elaborated by normal dog cells, we next examined the possibility that dogs with cyclic hematopoiesis had alterations in production or responsiveness to GM-CSA. Because lithium abolishes cyclic neutropenia in these dogs, we also examined the effect of lithium in vitro and in vivo on GM-CSA generation. Gray collie dogs responded to the GM-CSA normally (Table 1) and produced comparable quantities of GM-CSA when maximally stimulated (PHA = 50 µg/mL; Fig 4). During the time of "neutrophilia" (cycle days 8 through 12), the gray collie cells produced more GM-CSA with lithium and low doses of PHA than did normals. Inhibitor assays suggest this increased activity is not due to a decrease in an inhibitor. Furthermore, when a gray collie's cycling was eliminated by lithium treatment in vivo, this augmentation of GM-CSA production was not evident (Fig 5).

Studies of the mechanism of lithium-induced granulocytosis have shown that marrow cell production is increased,²⁵ and that lithium can stimulate the production of "CSA," the production of factors other than "CSA," and the proliferation of primitive granulocytic progenitor cells by a direct although unspecified mechanism.²⁶⁻³² Lithium prevented the granulopoietic differentiation arrest caused by human leukocyte interferon and increased physical interactions between monocytes and T lymphocytes, suggesting still other possible mechanisms of action.^{33,34} Our prior studies of the mechanism of lithium normalization of cyclic hematopoiesis failed to show any alteration in "CSA" in serum¹² and in this study we did not detect differences in GM-CSA generation by mononuclear cells with lithium added in the absence of PHA. However, lithium was effective in increasing GM-CSA at precisely the time the marrow neutrophilic precursors are at their nadir, suggesting that this mechanism could be physiologically relevant.

Two other lines of evidence suggest that these findings may be important. Recently, Jones et al have shown that bone marrow adherent cells fail to produce a stimulatory factor on cycle day 9 in their dogs with cyclic hematopoiesis and that marrow cell conditioned media inhibit murine CFU-S proliferation.^{35,36} Their data do not suggest that a

Table 4. Effect of Cyclosporine on GM-CSA Production by Dog Mononuclear Cells

				Colonies per 10 ⁵ Cells
Controls				
1	5% NDS without additives			15 ± 1
	5% NDS with 10% PHA-LCM			
2	(control)			60 ± 16
Culture Additives				
Test LCMS	5% NDS	PHA	Li*	CSP†
1	+	—	—	—
2	+	+	—	—
3	+	+	+	—
4	+	+	+	+
5	+	+	—	+
6	+	—	—	+

Colony numbers are means of triplicate plates (±SD) from normal dog bone marrow cells stimulated by additives as indicated. PHA, 50 µg/mL added to mononuclear cells to produce PHA-LCM. NDS, normal dog serum.

*Lithium chloride, 2 mmol/L (2 mEq/L).

†Cyclosporine, 3 µg/mL.

‡Different from test LCM 3 ($P < .05$) but not different from control 1 ($P > .30$).

lymphoid effector cell is involved, but certainly do not rule out the possibility, since the adherence procedure may trap lymphocytes as well as monocytes. The published data suggest that a lack of neutrophilic progenitor cells at the time of normal neutrophil counts is the proximate cause of the subsequent neutropenia in this disorder; a therapy that blocks an aberrant inhibitor or provides an absent stimulator at this phase of the cycle makes an attractive hypothesis. In addition, Verma et al reported studies in a patient with acquired cyclic neutropenia who responded to lithium treatment with improvement in her neutrophil counts.³⁷ In this person they implicated blocking of T lymphocyte suppression of monocyte CSA generation as being important in the lithium response.

We conclude from these studies that PHA is a useful stimulator of GM-CSA in dogs, as it is in mice and humans.

Using this form of GM-CSA, we have shown that gray collie and normal marrow cells respond by producing similar numbers and subtypes of CFU-c. In addition, the capacity of gray collie cells to increase GM-CSA levels in response to Li during the period of normal neutrophil counts is greater than that of normal dogs or gray collies treated *in vivo* with Li. These data suggest that Li-induced normalization of cyclic hematopoiesis in the gray collie could be due to intramedullary generation of GM-CSA at the critical time of deficient neutrophilic progenitor cell populations in the marrow.

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The Xerocytosis of Hb SC Disease

By Samir K. Ballas, James Lerner, Eugene D. Smith, Saul Surrey, Elias Schwartz, and Eric F. Rappaport

Patients with Hb SC disease were found to have microcytic and hyperchromic red cell indices despite mild reticulocytosis. Iron deficiency anemia was ruled out by the finding of normal serum ferritin levels. In order to determine whether the microcytosis was due to coexistent α -thalassemia, restriction endonuclease mapping was performed on genomic DNA extracted from peripheral blood leukocytes. Patients with Hb SC disease had microcytic indices despite the presence of a full complement of four α -genes ($\alpha\alpha/\alpha\alpha$), suggesting that the microcytosis may be due to cellular dehydration (or xerocytosis), since the mean corpuscular hemoglobin concentration in Hb SC disease patients was significantly higher than in controls. This possibility was investigated further by the determination of RBC cation content. RBC Na levels were similar in SC and normal red cells. Hb SC RBCs, however, had significantly reduced K levels. These findings show that RBC cation content, and

thus cell water, is decreased in Hb SC disease. The decreased RBC K level in the presence of normal cellular Na concentration suggests selective K loss that is not due to inhibition of the Na K pump. Ouabain-insensitive K^+ efflux was increased to four times normal in SC cells. Cell dehydration was confirmed by the demonstration of increased high-density RBCs on discontinuous Stratan density gradients and by osmotic gradient ektacytometry. Cellular dehydration and its sequelae were worse in CC erythrocytes and milder in AC cells than in Hb SC red cells. Taken together, these data indicate that in Hb SC disease the RBCs are severely dehydrated and typically microcytic and hyperchromic. Hb SC RBCs seem to be dehydrated due to selective K loss. These findings suggest a functional interrelationship between Hb SC, the red cell membrane, and cation regulation.

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THE ANEMIA of Hb SC disease is mild and often described as normochromic and normocytic, with many target cells and "boat-shaped" or "fat" sickle cells in the peripheral smear.^{1,2} We³ and others,⁴⁻⁶ however, reported that patients with Hb SC disease typically have microcytic and hyperchromic erythrocytes. Furthermore, Fabry et al⁷ found an unusually high mean corpuscular hemoglobin concentration (MCHC) in Hb SC disease that seems to result from cellular dehydration (xerocytosis). Bunn et al⁸ also noted an abnormally high MCHC in SC cells and suggested that the presence of Hb C resulted in the high MCHC of Hb SC cells early in their lives. These studies suggest that the high MCHC may be an important contributory factor to the pathogenesis of Hb SC disease. This study was designed to elucidate the basis of cellular dehydration in Hb SC erythrocytes obtained from our current patients with this disease and determine the rheological and pathophysiologic consequences of this phenomenon.

MATERIALS AND METHODS

Patients. All patients were examined and diagnosed at the Sickle Cell Center of the Cardeza Foundation of Jefferson Medical College and Thomas Jefferson University Hospital during the past 10 years (1975 to 1985). A total of 35 patients with Hb SC disease was seen during this period. The procedures described in this paper, however, were performed on blood obtained from 16 individuals with

$\alpha\alpha/\alpha\alpha$ genotype and Hb SC disease who were being followed in our clinic while this study was in progress. All patients were American blacks. Averages of the numerous hematologic data obtained on each patient were determined. Hematologic data on transfused patients were included in this study only if determined at least 4 months after the last transfusion. Only one patient in this study was a pediatric subject, a 6-year old girl; all others were adults whose ages ranged from 20 to 60 years. Five of these patients were males. Both males and females had similar mean corpuscular volume (MCV), MCHC, and reticulocyte values, but the hemoglobin and hematocrit levels were higher in males than in females, as was previously reported.³ The three individuals with Hb AC trait were females whose ages ranged from 20 to 25 years. The patient with Hb CC disease was a 9-year old female. Control subjects were black university personnel or normal adult black individuals who came to our clinic to determine whether they were carriers of the sickle gene.

Erythrocyte preparation. Erythrocytes from patients and controls were drawn into heparin or acid-citrate-dextrose, cooled to 4 °C, and processed as follows: hemoglobin, hematocrit, red cell count, reticulocyte count, red cell electrolytes, and DNA extraction were performed on freshly drawn samples within one to two hours of collection. Cation flux experiments and ektacytometry were determined within 24 hours of collection. Other procedures were done within 24 to 48 hours of collection or as indicated. Sickle red cells suspended in citrate, phosphate, and dextrose do not exhibit a density change for five days,⁹ and therefore maintain the pattern of their distribution on discontinuous Stratan density gradients during this period. All procedures, unless otherwise noted, were performed at 4 °C. Aliquots of cells were washed at least three times in buffers as specified for the various procedures described below, and the buffy coat was removed by aspiration after each wash.

Hematologic data. Hemoglobin and reticulocyte determinations were made by routine methods and the microhematocrit was obtained by centrifugation. Red cells were counted on a Coulter Model F cell counter (Coulter Electronics, Hialeah, FL). Serum ferritin was determined by a radioimmunologic assay.¹⁰

Hemoglobin electrophoresis on starch gel, starch block, or agar gel was performed on membrane-free hemolysates by established methods.¹¹ Hemoglobin solubility testing was conducted by using a commercial assay (Sickledex; Ortho Diagnostics, Raritan, NJ). Individual globin chains were differentiated by cellulose acetate electrophoresis of membrane-free hemolysate as described by Ueda and Schneider.¹²

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Determination of α -globin genotype. Alpha-globin genotypes ($\alpha\alpha/\alpha\alpha$, and $-\alpha/\alpha\alpha$) were determined by Southern blot hybridization restriction endonuclease mapping¹³ of genomic DNA extracted from peripheral blood leukocytes. The restriction endonucleases used were *Bam*HI and *Bgl*II, and the probe was *Bam*HI linearized α -cDNA plasmid JW 101¹⁴ that was ³²P-labeled with calf thymus DNA primers.¹⁵

Ektacytometric studies. The ektacytometer, a visco-diffractometer, designed and previously described by Mohandas et al^{16,17} was used to determine red cell deformability under various experimental conditions. This device imposes a well-defined laminar shear stress field on erythrocytes while simultaneously monitoring the extent of cell deformation. The instrument consists of a concentric cylindrical viscometer in which one cylinder can be rotated to produce the desired level of shear stress. A laser beam is directed through the cell suspension that is contained within a 0.5-mm gap between the two cylinders. The cells, oriented by the shear stress field, then diffract the laser beam to produce a coherent diffraction pattern, from which cell deformation is determined, using a photometric image analysis system.^{16,17} A "deformability index" (DI) is obtained that is equivalent to a measure of the ellipticity of a uniformly deforming cell population. In the standard mode of operation, the DI is recorded continuously as a function of shear rate.^{17,18} For measurement of intact cell deformability, 10 μ L of 40% red cell suspension are thoroughly mixed with 10.0 mL of polyvinylpyrrolidone (PVP; molecular weight 360,000 4 g/dL wt/vol, 97.5 cp, 290 mosm/kg, pH 7.4). This suspension produces a maximum shear stress of 170 dynes/cm² at the maximum applied shear rate of 100 rpm. Numerical values of the maximum deformability index reached, defined as DI max, were used to compare deformability of different samples.

The ektacytometer was also used to measure whole cell deformability of erythrocytes as a continuous function of the suspending medium osmolality at a constant applied shear stress of 170 dynes/cm² (osmotic gradient ektacytometry). For these studies the DI of red cells was continuously recorded as the suspending medium osmolality was increased from 50 to 700 mosm/kg as previously described.¹⁹

Separation of red cells by density. Subpopulations of erythrocytes of uniformly defined densities were isolated on discontinuous Stractan II (Champion International, Tacoma, WA) density gradients by centrifugation of normal and Hb SC cells.²⁰ The gradients covered a density range from 1.065 to 1.139 g/mL, in increments of 0.004 g/mL.

Active and passive influx of ⁸⁶Rb. Influx of ⁸⁶Rb into red cells was determined in the presence or absence of 0.1 mmol/L ouabain as previously described.^{21,22}

Potassium efflux. Red cells were washed three times in a HEPES-buffered (10 mmol/L pH 7.50) NaCl solution (290 mosm/kg) containing 5 mmol/L glucose, and the buffy coat was removed after each wash. Washed cells were resuspended at a hematocrit of 10% in the same medium, containing 0.1 mmol/L ouabain to define the ouabain-insensitive component of K⁺-efflux. After preincubation at 37 °C for 10 minutes, triplicate aliquots of cell suspension were removed at 30-minute intervals, centrifuged in a microfuge,

and the supernatants removed for subsequent analysis of K⁺ by flame photometry. Initial intracellular cation concentrations were determined on hemolysates of cell aliquots washed in isotonic Tris-buffered MgCl₂ (10 mmol/L Tris-HCl, pH 7.4, 290 mosm/kg). First-order K⁺ efflux rate constants were determined by plotting the log of the declining intracellular K⁺ concentrations, calculated from the initial and supernatant concentrations, versus time.²²

Adenosine triphosphatase (ATPase) assays. Hemoglobin-free erythrocyte membranes were prepared from red cells by hypotonic lysis and washing in 7 mmol/L NaCl, 5 mmol/L Tris-HCl, 1.0 mmol/L EDTA, pH 7.4.

The Mg⁺⁺-dependent ATPase activity was assayed in a medium containing 3.0 mmol/L ATP (disodium salt neutralized to pH 7.1), 20 mmol/L Tris-HCl, pH 7.4, 1.0 mmol/L EGTA, 0.2 mmol/L ouabain, 3 mmol/L MgCl₂, 116 mmol/L NaCl, and 16 mmol/L KCl. Ouabain was omitted when the total Na⁺-K⁺ ATPase activity was to be determined. The Na⁺-K⁺ ATPase was defined as the ouabain-sensitive component, and the ouabain-insensitive component as the Mg⁺⁺-dependent ATPase activity. Each assay tube contained 100 to 300 g of membrane protein in the presence or absence of 0.2 mmol/L ouabain in a total volume of 2.0 mL. ATP and membrane blanks were run simultaneously with incubations at 37 °C for one hour. ATPase activity was expressed as nmoles Pi liberated per milligram membrane protein per/minute by the method of Fiske and Subbarow²³ as modified by Bartlett.²⁴

Analytical methods. Red cell sodium and potassium concentrations were determined by flame photometry of red cell aliquots washed in ice-cold, isotonic Tris-buffered MgCl₂ solution (10 mmol/L Tris-HCl, pH 7.4). Red cell membrane protein content was determined by the method of Lowry et al.²⁵

RESULTS

Hematologic data. Hematologic data of the 16 patients studied with Hb SC disease and $\alpha\alpha/\alpha\alpha$ genotype are summarized in Table 1. The MCV in Hb SC disease is decreased ($P < .001$) despite mild reticulocytosis, while the MCHC is increased ($P < .001$). Iron deficiency was ruled out by the finding of normal or elevated serum ferritin in 12 patients with Hb SC on whom ferritin levels could be obtained. Thus, the red cell indices in Hb SC disease are typically microcytic and hyperchromic. Similarly, individuals with Hb AC and CC and four α -genes also have microcytic and hyperchromic indices with no iron deficiency.

α -Globin genotype. Restriction endonuclease mapping of genomic DNA showed that the 16 patients with Hb SC disease, the three individuals with Hb AC, and the patient with Hb CC had normal α -globin genotype ($\alpha\alpha/\alpha\alpha$).

Red cell cation content. The hydration status of Hb, SC, AC, and CC erythrocytes was studied by determining the red cell cation content, cell distribution on discontinuous Stractan density gradients, and osmotic gradient ektacytometry.

Table 1. Hematologic Parameters of Controls and Individuals With Four α -Genes and Hb SC, AC, or CC

Parameter	Controls	Hb SC	Hb AC	Hb CC
MCV (fl)	(11) 92.4 $\pm$ 5.14	(16) 79.5 $\pm$ 4.45	(3) 79.1 $\pm$ 3.96	(1) 55.3*
MCHC (g/dL)	(11) 32.9 $\pm$ 1.40	(16) 35.9 $\pm$ 0.84	(3) 35.4 $\pm$ 1.10	(1) 35.6*
Reticulocytes (%)	(11) 1.8 $\pm$ 1.55	(16) 4.9 $\pm$ 1.31	(3) 2.6 $\pm$ 1.76	(1) 6.8*
Ferritin (ng/mL)	>20	(12) 25-680	(1) 53	(1) 76

Values shown are mean $\pm$ SD. Number of individuals on whom the indicated parameter could be performed is indicated in parentheses.

*Number is average of five determinations.

Table 2. Cation Concentration and Content of Normal and Hb SC, AC, and CC Cells

RBC	mEq/L RBC			mEq/10 g Hb		
	Na ⁺	K ⁺	Na ⁺ + K ⁺	Na ⁺	K ⁺	Na ⁺ + K ⁺
Control (11)	7.2 ± 1.35	103.2 ± 4.42	110.4 ± 5.05	0.22 ± 0.04	3.15 ± 0.21	3.37 ± 0.23
Hb SC (16)	9.6 ± 2.95	89.5 ± 5.16	99.2 ± 3.13	0.26 ± 0.08	2.50 ± 0.15	2.76 ± 0.12
Hb AC (3)	6.5 ± 1.08	100.2 ± 2.08	106.6 ± 2.72	0.19 ± 0.03	2.86 ± 0.10	3.04 ± 0.11
Hb CC *(1)	9.0	84.0	93.0	0.25	2.48	2.73

Values shown are mean ± SD. Number of determinations is indicated in parentheses.

*Values are averages of three determinations.

The cation content of normal and abnormal erythrocytes is summarized in Table 2. The Na content of abnormal cells was similar to controls, while the K content was significantly decreased. Noteworthy is that Hb SC and CC erythrocytes had the lowest K⁺ level, whereas AC cells had a K⁺ level that was intermediate between control and SC and CC RBC.

Distribution of Hb SC cells by density. Cellular dehydration in Hb SC, AC, and CC cells was confirmed by the presence of increased high-density cells after centrifugation on discontinuous Stratan density gradients (Fig 1). There is a shift in SC and CC cell population to higher density. Hb AC cells, again, are intermediate between controls on the one hand and SC and CC cells on the other hand.

Monovalent cation fluxes in SC cells. Various components of monovalent cation fluxes were measured to provide further information about cation regulation in Hb SC cells. ⁸⁶Rb influx in the presence or absence of 0.1 mmol/L ouabain indicated that the total influx of Rb was moderately increased in Hb SC cells while the ouabain-insensitive (passive) influx was similar in control and Hb SC RBC (Fig 2). The ouabain-sensitive influx of Rb, which represents the active pump-mediated component, was also moderately increased in SC cells as compared with controls. Measurement of ATPase activity in membrane preparations from SC cells showed mildly increased Na-K ATPase and Mg ATPase activities (Table 3).

In order to investigate the nature of the abnormality in cation regulation in Hb SC erythrocytes further, we determined the ouabain-insensitive K⁺ efflux in the presence or absence of 1.0 mmol/L piretanide or furosemide, inhibitors of Cl⁻ transport and chloride-dependent K⁺ flux. Figure 3 shows that SC cells had a markedly increased K efflux. The first-order rate constant for K efflux for SC cells was 0.043 h⁻¹, compared with 0.012 for control cells (258% increase). The addition of 1.0 mmol/L piretanide or furosemide to the medium reduced the rate constant for K efflux of normal cells by 16% to 20%, respectively, but did not decrease that of SC cells (Table 4). Table 4 also shows that K⁺ efflux from CC erythrocytes was even greater than that from SC cells. AC erythrocytes showed K⁺ efflux values that were intermediate between SC and control red cells. Again the addition of piretanide or furosemide did not reduce the K⁺ efflux from AC and CC cells.

Ektacytometric studies. In order to further elucidate the hydration status and surface area-volume ratio in SC, AC, and CC erythrocytes and their subpopulations, we studied these cells by osmotic gradient ektacytometry as previously described.¹⁹ Figure 4A shows the deformability profile of

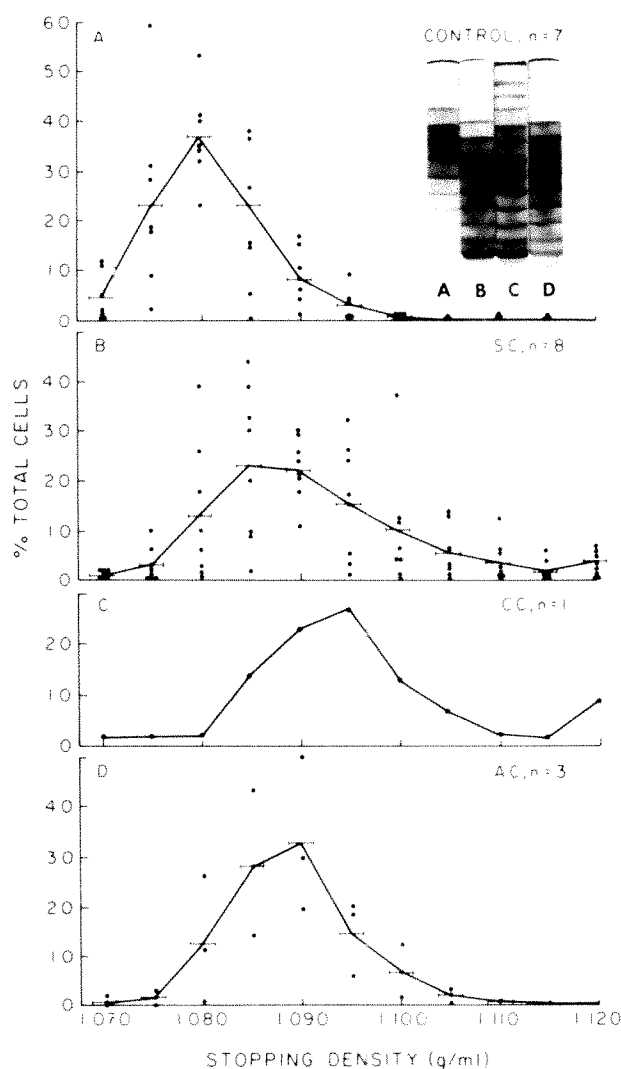


Fig 1. The density of RBC from individuals with Hb Sc. The percentage of RBC from fractions of the analytical Stratan gradients is shown on the Y-axis and the densities of the gradient layers on which each fraction stopped are shown on the X-axis. RBC are from (A) control subjects with Hb A and four α -globin genes, (B) Hb SC patients with four α -globin genes, (C) a patient with Hb CC disease and four α -globin genes, and (D) Hb AC individuals and four α -globin genes. The horizontal lines indicate the mean value for each set of values. The inset shows representative actual density profiles from (A), (B), (C), and (D) described above.

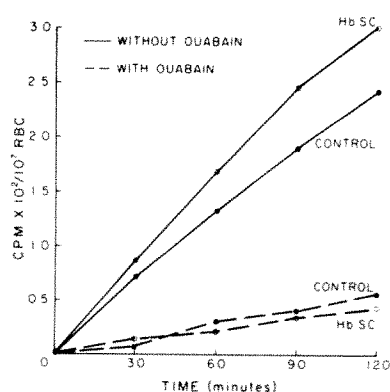


Fig 2. ^{86}Rb influx into control and Hb SC red cells. Erythrocytes were incubated at 37°C in glucose phosphate-buffered saline containing 5 mmol/L K and ^{86}Rb in tracer quantities, in the presence or absence of 0.1 mmol/L ouabain.

total SC cells from patients with four α -genes. Although the DI values of SC cells at 290 mosm are less than those for control cells, there is a wide range of variation in the shape of the curve among the patients studied. The range shown in Fig 4A is based on 40 ektacytometric studies on cells from 16 patients with four α -genes. The shape of the curve for each patient was the same when determined on blood samples drawn at different time periods. The curves of all patients were substantially shifted to lower osmolality compared with normal curves. This finding was shown previously to indicate red cell dehydration.^{17,19} Figure 4B shows the deformability profile of erythrocytes from three individuals with Hb C trait and four α -globin genes and one patient with Hb CC and four α -globin genes. Hb CC erythrocytes showed severe dehydration as indicated by shift of the curve to very low osmolality. The curves of Hb AC cells indicate less severe dehydration than those of CC erythrocytes, and they fall within the range of the curves for SC cells shown in Fig 4A.

Because the average maximum DI of SC cells represents a heterogeneous mixture of cells covering a broad MCHC range, we next obtained osmotic gradient deformability profiles on erythrocyte subpopulations of the same density isolated on preparative Stractan gradients from one patient with Hb SC disease, another with Hb CC, and a third with Hb AC. Figure 5 shows that all curves are shifted to lower osmolality compared with the normal range. Each Stractan fraction is shifted to lower osmolality, and the degree of this shift increases as one moves from AC to SC and CC cells. Thus Hb CC subpopulations have the lowest DI and are shifted to lowest osmolality values. Table 5 also shows that the cation content of each Stractan fraction decreases as one

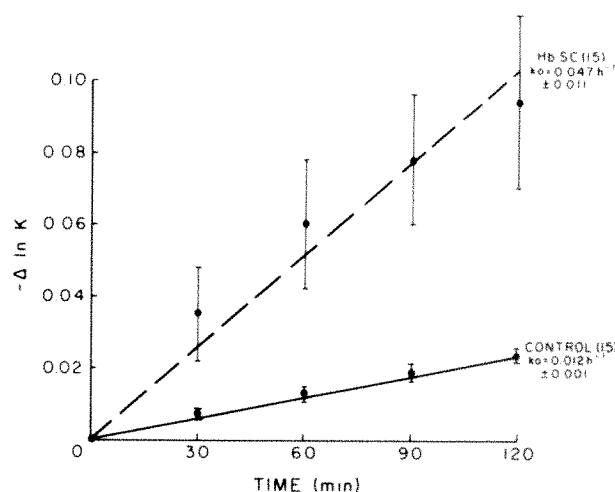


Fig 3. Kinetics of ouabain-insensitive K^+ loss from freshly drawn control and Hb SC red cells. Erythrocytes were incubated at 37°C in 10 mmol/L HEPES, pH 7.5, 5 mmol/L glucose, 0.1 mmol/L ouabain, and NaCl to bring the final osmolality to 290 mosm/kg. At intervals, aliquots were removed, K^+ concentration in supernatants were determined, and the first-order rate constants for K^+ efflux were calculated.

compares normal, SC, AC, and CC erythrocytes. Hb AC subpopulations have a total cation content that is intermediate between control RBC and other variant cells. Although Hb CC fractions seem to have slightly higher K^+ content than do SC cells, they are nevertheless more dehydrated than SC RBC. We attribute this difference to their much higher reticulocyte count, which, because of the lower cell Hb content, gives a higher K^+ level when it is expressed relative to Hb content. Noteworthy in Fig 5 is that reticulocyte-rich RBC fractions (density = 1.070) had lower maximum deformability than was expected for these low-density RBC.

DISCUSSION

In this study we have found that erythrocytes in Hb SC disease are dehydrated and typically microcytic and hyperchromic. The combination of relatively low MCV and an increased MCHC is unusual and seems to be characteristic of Hb SC disease.³⁻⁶ We must emphasize that red cell indices were determined by using manual methods for the determination of hemoglobin and hematocrit and the Coulter Counter Model F for the estimation of the red cell count. Mohandas et al²⁶ have shown that for MCHC values above 36 g/dL as measured by the spun hematocrit method, electronic counters (Coulter S, Coulter S-Plus, and Ortho ELT-8) underestimated the MCHC with increasing error as the true MCHC increased. The choice of the methods to determine red cell indices may explain why some reports²⁷ did not emphasize the hyperchromic nature of RBC in Hb SC disease. Fabry et al⁷ have shown that the MCHC in Hb SC disease is unusually high by using a Percoll-Stractan continuous-density gradient. They found that Hb SC cells are much denser than normal cells, and they estimate an average MCHC of 36.4 ± 0.76 g/dL on the basis of the cell distribution on the gradient. Bunn et al⁸ determined the

Table 3. ATPase Activities in RBC From Controls and Individuals With Hb SC, AC, or CC

	Na ⁺ - K ⁺ ATPase Activity (nmoles Pi/mg/min)	
	Ouabain-Sensitive	Ouabain-Insensitive
Control	(5) 7.3 ± 1.16	(5) 4.0 ± 0.78
Hb SC	(8) 10.8 ± 4.99	(8) 5.8 ± 1.41
Hb AC	(4) 6.3 ± 1.71	(4) 3.93 ± 1.21
Hb CC	(1) 12.8	(1) 7.58

Table 4. Effect of Piretanide and Furosemide on Ouabain-Insensitive K⁺ Efflux From Normal and Hb SC, AC, and CC Cells

Additive	-Δ ln K Conc/mL Cells			
	Normal	Hb SC	Hb AC	Hb CC
None	(11) 0.025 ± 0.0018	(11) 0.100 ± 0.0263	(9) 0.037 ± 0.0065	(4) 0.151 ± 0.0353
1.0 mm piretanide	(4) 0.021 ± 0.0029	(3) 0.144 ± 0.0265	(2) 0.051 ± 0.0148	(2) 0.177 ± 0.0290
1.0 mm furosemide	(3) 0.020 ± 0.0030	(5) 0.117 ± 0.0258	(5) 0.036 ± 0.0023	(1) 0.174

Cells were incubated for two hours at 37 °C in 10 mol/L HEPES (pH 7.5), 5 mol/L glucose, 0.2 mol/L ouabain, and NaCl to bring the final osmolality to 290 mos/kg, in the presence or absence of the indicated additive. Values shown are the negative 1n of the calculated intracellular K concentration as a function of time. Values shown are mean ± SD. Number of determinations is indicated in parentheses. Results were corrected for the small amount of hemolysis that occurred during incubation.

distribution of AA, AS, SC, AC, and CC RBC on discontinuous Stractan gradients and found increased proportions of dense cells whenever Hb C was present. Our findings agree with these previous reports and furnish new data on Hb SC RBC from a relatively large number of patients that include passive K permeability and pump activity, Na-K ATPase, rheologic measurements that reflect the properties of individual cells (as opposed to bulk viscometric methods), and deformability measurements on Stractan gradient subpopulations.

Microcytosis in Hb SC disease seems to be secondary to cellular dehydration. This was demonstrated by the finding of decreased cation content of SC cells, by the presence of a high percentage of dense cells on Stractan gradients, and by osmotic gradient ektacytometry. Dehydration of SC cells seems, in turn, to be due to high and selective efflux of cellular K⁺ that is not inhibited by ouabain, piretanide, or furosemide. This severe dehydration results in decreased cell volume and increased surface area-volume ratio, which explains the decreased osmotic fragility of SC cells shown by osmotic gradient ektacytometry. Thus AC red cells are least affected and CC cells are the most dehydrated. Brugnara et al²⁸ have recently reported some similar findings in RBC homozygous for Hb CC. These cells were found to contain less cations and water than did normal RBC due to an increase in K⁺ efflux that was insensitive to ouabain and bumetanide, but dependent on cell volume and internal pH.

Microcytosis in Hb SC disease does not appear to be secondary to coexistent iron deficiency anemia or α-gene

deletion, since serum ferritin levels were within the normal range or elevated in all patients on whom this test was determined. Patients with four α-genes had microcytic and hyperchromic indices. Glader et al²⁹ also indicated that even in sickle cell anemia there is "relative microcytosis," since the MCV values in this disease are less than expected for the degree of reticulocytosis. An unusual observation in this study is that reticulocyte-rich SC fractions had lower DI than expected from their low MCHC. A similar observation in reticulocyte-rich fractions from sickle cell erythrocytes was reported by Kaul et al.³⁰ The mechanisms responsible for this observation are unknown. Lack of membrane remodeling due to functional asplenia in these patients may be one possibility.

Factors responsible for the K⁺ efflux in Hb SC disease are not known. One possibility may relate to the high affinity of Hb C for the erythrocyte membrane.³¹ Preferential binding of β^C chains to red cell membranes in comparison to β^A and β^S globin polypeptides has been reported.^{32,33} The high affinity of Hb C for the red cell membrane seems to be similar to that reported for the glycolytic enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase.^{34,35} It is believed that hemoglobin binds to the cytoplasmic portion of band 3 of the red cell membrane,³⁶⁻³⁸ since it competes with the binding of glycolytic enzymes to the membrane. Binding to the membrane in each case is specific and primarily electrostatic, since it is highly sensitive to ionic strength and pH.^{34,39} Moreover, the composition of the NH₂-terminal of band 3 in human cells is very acidic. The first 31 amino acid residues

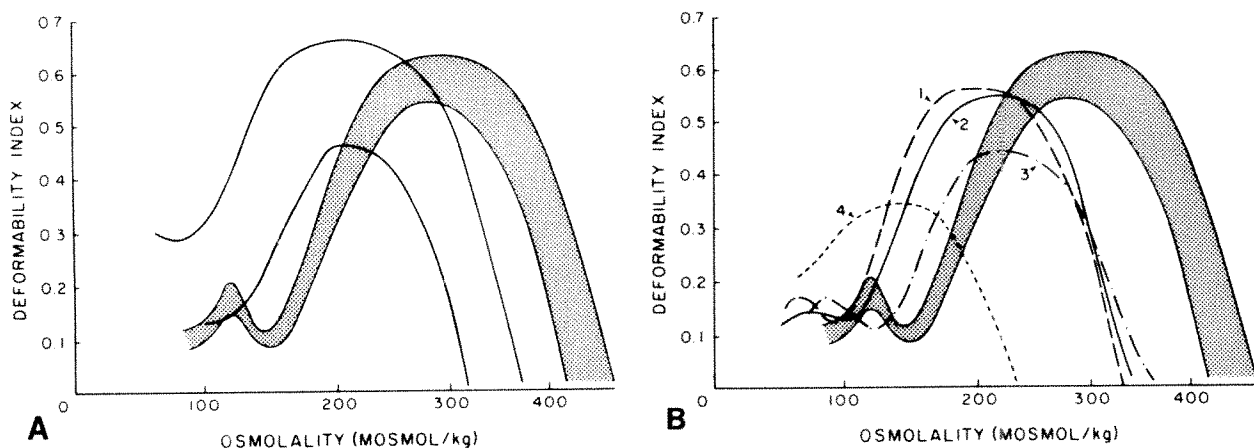


Fig 4. Osmotic deformability profile for control and Hb SC red cells. The shaded area represents the range for normal controls. (A) represents the range of 40 determinations on RBC from 16 patients with Hb SC disease and (B) represents deformability profiles on subjects with Hb AC (Nos. 1, 2, 3) and Hb CC disease (No. 4). All individuals had αα/αα genotype.

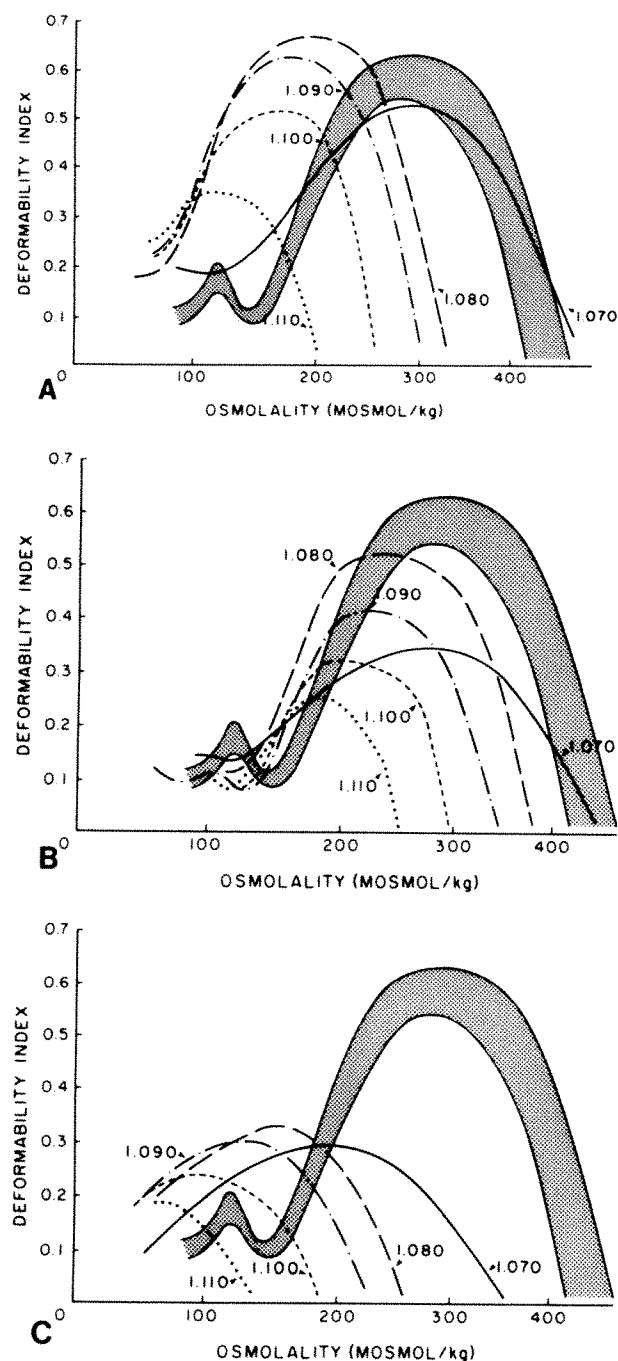


Fig 5. Osmotic deformability profiles for RBC subpopulation isolated on Stractan density gradients. Numbers on the hypertonic arm of the curves indicate the density of the Stractan layer above which the RBC population rested. The shaded area represents the range for normal controls. RBC are from (A) Hb SC patient, (B) Hb AC individual, and (C) Hb CC patient.

contain 16 aspartic and glutamic acid residues, no basic residues, and a blocked α -amino group.^{40,41} This composition may explain the electrostatic mode of band 3 binding to Hb C, presumably to the basic lysine residue, which replaces glutamic acid normally present at the sixth position of the β -polypeptide chain.

Table 5. Properties of Normal, SC, AC, and CC RBC Subpopulations Isolated by Stractan Gradient Centrifugation

RBC	Stopping Density*	Reticulocyte (%)	MCHC (g/dL)	Cations mEq/10 g Hb		
				Na ⁺	K ⁺	Na + K
Control	1.080	2.6	32	0.25	3.16	3.41
	1.090	1.7	34	0.25	2.77	3.02
	1.100	0.9	37	0.33	2.42	2.75
	1.110	—	—	—	—	—
SC	1.080	4.5	34	0.22	2.74	2.94
	1.090	2.1	38	0.21	2.38	2.59
	1.100	1.2	40	0.26	1.68	1.94
	1.110	0.3	44	0.22	1.18	1.40
AC	1.080	2.1	33	0.27	2.89	3.16
	1.090	0.9	38	0.25	2.48	2.73
	1.100	0.5	40	0.32	2.17	2.49
	1.110	0	—	—	—	—
CC	1.080	10.5	35	0.22	2.82	3.04
	1.090	7.8	38	0.20	2.33	2.53
	1.100	6.2	39	0.26	1.82	2.08
	1.110	5.1	42	0.35	1.24	1.59

*Density of the Stractan layer on which each cell subpopulation rested.

Taken together, our data suggest a functional interrelationship among Hb SC, membrane stability, and cation regulation. The pathophysiologic sequence of events in Hb SC disease may be as follows: Hb SC interacts with the membrane (probably band 3), somehow increasing K⁺ efflux. Cellular dehydration ensues, with concomitant increase in MCHC and abnormal rheological properties. Microcytosis is probably also a consequence of cellular dehydration. As has been shown for Hb CC cells,²⁸ the cell membrane permeability may be modulated by the hydration status of Hb SC RBC. Thus the more dehydrated the cells, the greater is their K⁺ efflux due to increased membrane permeability. This sequence of events seems to be milder in AC cells and worse in CC erythrocytes than in SC red cells. The exact mechanisms involved in this sequence of events remain to be determined.

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7q32-q36 Translocations in Childhood T Cell Leukemia: Cytogenetic Evidence for Involvement of the T Cell Receptor β -Chain Gene

By Susana C. Raimondi, Ching-Hon Pui, Frederick G. Behm, and Dorothy L. Williams

Blast cell chromosomal rearrangements involving the long arm of chromosome 7 were identified in eight of 197 cases of childhood acute lymphoblastic leukemia (ALL). Breakpoints were variable but tended to cluster in either the proximal or the terminal 7q region, depending on the immunophenotype of the cells. The 7q32-q36 region, the locus of the T cell receptor β -chain gene, was the site of breakpoints in four of 31 cases of T cell ALL but was not involved in any of the 166 cases originating from B cell precursors ($P < .0004$). In three of the four T cell cases it was possible to identify the chromosomal segment that had been translocated to the 7q32-q36 region: 1p32, 2p21,

and 6p21. The 1p32 and 6p21 bands are particularly interesting, as they contain the sites of two known proto-oncogenes, *c-L-myc* and *hpim*, respectively. Our findings suggest that the locus of the β -chain gene of the T cell receptor is a preferential site for certain chromosomal rearrangements in leukemic T lymphoblasts, analogous to the T cell receptor α -chain gene on human chromosome 14. Translocation of proto-oncogenes to a site near the β -chain regulatory sequences provides a potential mechanism for oncogene activation.

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CERTAIN CHROMOSOMAL translocations are specific for particular subtypes of human leukemia and lymphoma.¹ The most striking example can be found in B cell neoplasias, in which translocations involve chromosomal regions containing the immunoglobulin (Ig) kappa, mu, and lambda chain genes.^{2,3} These rearrangements consistently bring the *c-myc* proto-oncogene, on chromosome 8(q24), to a position adjacent to a rearranged and transcriptionally active Ig gene, leading to aberrant activation of *c-myc*.⁴ This model of gene activation has motivated a search for other instances of consistent chromosomal rearrangements that bring an "inactive" oncogene near "activating" sequences elsewhere in the genome.⁵

The organization and rearrangement of T cell receptor (TCR) genes during T cell differentiation are analogous to the changes seen in Ig genes in B cell precursors.^{6,7} Three classes of TCR-specific cDNA clones (α , β , and γ) have been isolated.^{8,9} The gene encoding the α -chain of the TCR has been mapped to bands q11-q12 of chromosome 14,^{10,11} a locus that is nonrandomly involved in translocations and inversions in human T cell neoplasias.¹²⁻¹⁷ Moreover, molecular alterations of the α -chain locus have been demonstrated in blast cells with the t(11;14)(p13;q13) in two cases of childhood T cell acute lymphoblastic leukemia (T-ALL)^{18,19} and in a T cell line with t(8;14)(q24;q11) derived from a child with T-ALL.²⁰ In a pediatric T cell leukemia line, inversion of chromosome 14 involving bands q11 and q32 resulted in the recombinational event of the Ig heavy-chain gene (14q32) with the gene for the TCR α -chain (14q11).^{21,22} These studies suggested that the α -chain gene locus may participate in proto-oncogene activation, in a manner similar to that seen in B cell lymphoid malignancies.^{4,5} The locus for the TCR β -chain gene has been assigned to region 7q32-q36 of chromosome 7.²³⁻²⁵ Recently, Brito-Babapulle et al²⁶ observed a strong correlation between the presence of a 7q abnormality and expression of the T cell growth factor receptor (Tac) in adult T cell leukemias, leading them to speculate that the chromosomal defect is indirectly responsible for activation of the Tac gene. Finally, 7p15, where the human gamma-chain genes of the TCR is located,²⁷ is a frequent site of chromosome breakage in ataxia telangiectasia, a T cell disorder.²⁸

We present cytogenetic evidence that translocations involving the q32-q36 region of chromosome 7 are nonran-

domly associated with childhood T-ALL and suggest that the TCR β -chain locus is affected by these chromosomal rearrangements.

MATERIALS AND METHODS

Patients. From August 1983 to December 1985, 227 consecutive children with newly diagnosed ALL were admitted to St Jude Children's Research Hospital. Thirty-three cases had a T cell immunophenotype, as judged from surface antigen reactivity with various T cell-related monoclonal antibodies. One hundred ninety-seven patients, including 31 with T-ALL, had blast cell metaphase preparations adequate for cytogenetic analysis. All patients were enrolled in studies that included a cell profile analysis, approved by the institution's clinical trials committee. All patients were advised of the procedures and attendant risks, in accordance with institutional guidelines, and informed consent was obtained in each instance.

Cytogenetic studies. Bone marrow samples, obtained from patients at the time of diagnosis, were processed according to the method of Williams et al.²⁹ Briefly, 0.1 to 0.2 mL of heparinized bone marrow was collected in tubes containing 8 mL of RPMI-1640 with L-glutamine, antibiotics, and 30% fetal calf serum. The cells were processed immediately after a 25-minute incubation in Colcemid (final concentration, 0.06 μ g/mL), rinsed twice in Hank's balanced salt solution, and exposed to a hypotonic solution (0.075 mol/L KCl) for a total of 32 minutes at room temperature, including periods of mixing, standing, and centrifugation. The cells were then

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fixed in methanol-acetic acid (3:1, vol/vol) for 15 minutes. Slides were prepared with use of a flaming technique and were allowed to age for zero to seven days. Metaphase preparations were G-banded by a modification of the trypsin method of Seabright,³⁰ and chromosomes were identified and assigned according to the International System of Human Cytogenetic Nomenclature.³¹

Immunophenotyping. Blast cells were separated on a density gradient, and only samples containing >85% blasts were assayed. Cell surface antigens were detected by a standard indirect immunofluorescence assay that relied on monoclonal antibodies to the major clusters of differentiation (CD) antigens described by the First and Second International Workshops on Human Leukocyte Differentiated Antigens.³² These included J5 (CD-10), anti-HLA-DR, OKT10, OKT9, 3A1 (CD-7), T101 (CD-5), T11 (CD-2), T3 (CD-3), T6 (CD-1), T4 (CD-4), T8 (CD-8), and B4 (CD-19). Two hundred cells were counted in each immunofluorescence assay; the results were considered positive if 40% or more of the blasts showed surface fluorescence. Cell surface immunoglobulin (sIg) was identified by a direct immunofluorescence assay with fluoresceinated goat antihuman immunoglobulin (Tago, Burlingame, CA). Cytoplasmic immunoglobulin (cIg) was identified by a direct method that used fluoresceinated F(ab')₂ goat antihuman μ -chains (Southern Biotechnology, Birmingham, AL).³³ Terminal deoxynucleotidyl transferase (TdT) was identified by use of an indirect immunofluorescence assay (Supertechs, Inc, Bethesda, MD).³⁴ The samples were classified phenotypically according to the patterns of reactivity as T cell (T⁺, E rosette⁺, HLA-DR⁺), common (CALLA⁺, HLA-DR⁺, E⁻, T⁻, cIg⁻, sIg⁻), pre-B cell (cIg⁺, HLA-DR⁺, CALLA⁺, E⁻, T⁻), or B cell (sIg⁺).

RESULTS

Chromosomal rearrangements involving the 7q arm were found in blast cells from eight of the 197 cases studied. The breakpoints in four of these cases occurred in the proximal 7q

Table 1. Clinical and Laboratory Data for Patients With T Cell Leukemia and 7q32-q36 Translocations

	Patient 1	Patient 2	Patient 3	Patient 4
Age (yr)	17	14	9	9
Sex	M	M	F	M
Mediastinal mass	Yes	Yes	Yes	Yes
Spleen size (cm)*	1	10	7	0
Liver size (cm)*	1	9	6	0
Hemoglobin (g/dL)	16.2	12.8	10.8	13.1
Leukocytes ($\times 10^9/L$)	12.4	233.5	13.2	1.6
Platelets ($\times 10^9/L$)	278	45	551	92
CNS leukemia	Yes	Yes	No	No
Surface markers†				
TdT	63	84	0	0
T101 (CD-5)	89	74	100	75
T11 (CD-2)	93	93	100	40
T3 (CD-3)	37	0	21	3
OKT9	59	74	100	75
OKT10	75	57	90	79
T6 (CD-1)	8	0	97	2
T4 (CD-4)	82	2	100	0
T8 (CD-8)	86	6	96	6
CALLA (CD-10)	28	74	0	0
HLA-DR	13	0	7	4

*Edge palpable below the costal margin.

†Cases were considered positive if $\geq 40\%$ of the cells reacted with the probe for a particular marker. The source of blasts was bone marrow from patients 1, 2, and 4 and pleural fluid from patient 3.

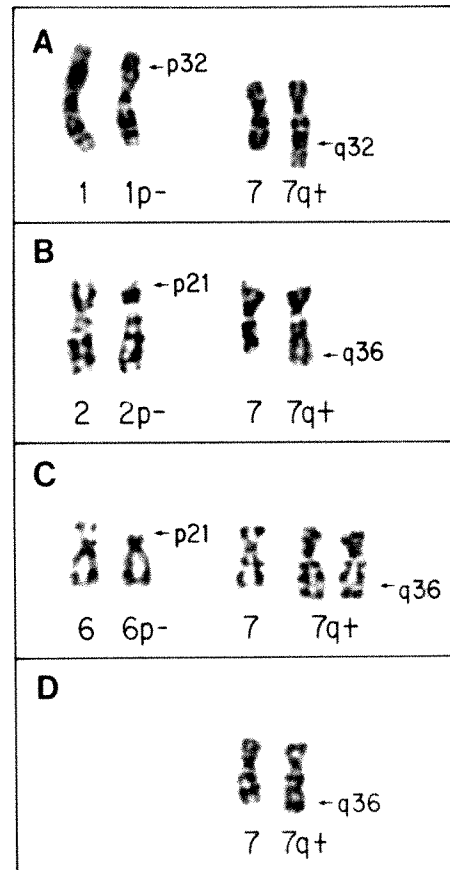


Fig 1. Different abnormalities involving the 7q32-q36 region in cases of T-cell leukemia. A through D show partial G-banded karyotypes of patients 1 through 4. The normal chromosomes of each pair are shown on the left and those for the rearranged chromosomes are shown on the right. Arrows indicate breakpoint sites.

region (two at 7q11 and two at 7q22), while in the remainder they were within the terminal 7q region (one at 7q32 and three at 7q36). The former group comprised three cases of common ALL and one of pre-B-ALL, whereas each of the latter four was classified as T cell ALL: common or mid-thymocyte stage (cases 1 and 3) and immature or early thymocyte stage (cases 2 and 4) (Table 1). Thus the terminal 7q region was involved in translocations within T lymphoblasts but not in those within non-T cells (four of 31 cases v zero of 166, $P < .0004$ by two-tailed Fisher's exact test). In T cell cases, the DNA material exchanged with the 7q32-q36 was different in each case (Fig 1 and Table 2), and none of the additional structural or numerical chromosomal abnor-

Table 2. Blast Cell Karyotypes

Patient No.	Karyotype
1	46,XY,t(1;7)(p32;q32),del(9)(p22)
2	46,XY,t(2;7)(p21;q36),del(5)(p14),del(9)(p21)
3	47,XX,t(6;7)(p21;q36),+der(7),t(6;7)(p21;q36)
4	44,XY,-5,-10,dup inv(1)(p32p13),t(6;7)(q27;?),t(7;7)(q36;?),t(16;7)(p13;?),t(9;13)(q11;p12)

malities were alike except for a deletion of the p arm of chromosome 9 in cases 1 and 2.

The clinical and laboratory features of patients with T cell ALL and a 7q32-q36 translocation are summarized in Table 1. These three boys and one girl ranged in age from 9 to 17 years (median, 11.5 years). All four had a mediastinal mass, and two had central nervous system leukemia at diagnosis. Patient 1 also presented with multiple scalp nodules and right facial paralysis, and patient 4 had an epidural tumor, resulting in paraparesis at diagnosis. Three of the patients entered complete remission and two remain free of leukemia for 10+ and 28+ months. Patient 3, who failed to achieve remission, was unusual in that there was a "lineage switch" from T cell ALL to acute myeloblastic leukemia after 3 weeks of induction therapy. At that time, cytogenetic analysis disclosed only normal diploid karyotypes.

DISCUSSION

Numerical abnormalities of chromosome 7 are common in hematologic disorders, but structural abnormalities of the 7q arm are identified much less often.¹ Cytogenetic analysis of 197 well-banded consecutive cases of childhood ALL in this study disclosed eight cases with rearrangements of the 7q arm. Breakpoints were variable but tended to show specificity for the immunophenotype of blast cells. In cases of T cell disease, breakpoints were restricted to the terminal 7q region (one at 7q32 and three at 7q36), whereas in B cell-precursor cases, they occurred in the proximal 7q region (two at 7q11 and two at 7q22).

The 14q11-q12 region, which contains the TCR α -chain gene,^{10,11} appears to be nonrandomly rearranged and presumably is involved in proto-oncogene activation in T cell neoplasias.^{13,18-22} Heretofore, the 7qter region (to which the TCR β -chain gene has been mapped) has received less attention as an important site of chromosomal abnormalities in T cell malignancies.^{1,35-37} Studying the blast cell chromosomes of a large number of childhood ALL cases has allowed us to demonstrate that 7qter abnormalities represent yet another subgroup of cytogenetic abnormalities that correlates with a

specific immunophenotype of ALL. In two of our cases, there was a loss of chromosome material from the short arm of chromosome 9(p21-p22), a finding previously reported in T cell lines,³⁷ as well as a distinct subtype of ALL characterized by "lymphomatous" features and presumably of T cell origin.³⁸ However, in a collaborative study, this chromosomal abnormality was found to bear no consistent relationship with blast cell immunophenotype or other presenting features.³⁹

Chromosome bands involved in each of the reciprocal translocations with the 7q32-q36 region were 1p32, 2p21, and 6p21. The 1p32 belongs to the region to which c-L-myc⁴⁰ has been mapped. Although no specific proto-oncogenes have been identified at 2p21, Brownell et al⁴¹ have recently isolated and localized human cellular *rel* sequences to chromosome 2. The related viral sequence, r-*rel*, was derived from the cellular genome of a turkey with leukemia induced by the reticuloendotheliosis virus. Finally, the oncogene *hpim* has been mapped to the 6p21 region.⁴² The *hpim* is a human homolog of a murine gene that is activated by retroviral insertion in murine T cell lymphomas. It seems reasonable to suggest that the c-L-myc, *hpim*, and other putative proto-oncogenes are deregulated as a consequence of their translocation to a position near the TCR β -chain gene and its regulatory sequences. Whether or not the gene encoding the β -chain of the TCR is involved in the genesis of childhood T cell ALL needs to be tested by molecular analysis of the DNA adjacent to breakpoints of the 7q32-q36 translocations.

NOTE ADDED IN PROOF

Since initial submission of the manuscript we have identified another T cell ALL case with a t(6;7)(q24;q36), strengthening the association of the 7qter region with T cell malignancies.

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Different Stimulative Effects of Human Bone Marrow and Fetal Liver Stromal Cells on Erythropoiesis in Long-Term Culture

By Ineke Slaper-Cortenbach, Rob Ploemacher, and Bob Löwenberg

The factors determining the predominantly erythroid direction of human fetal liver hematopoiesis are unknown. We compared the capacities of human fetal liver and bone marrow stromas to sustain fetal and adult hematopoiesis in long-term cultures. In various marrow-fetal liver combinations of stroma and recharge, the maintenance of erythroid (BFU-e) and myeloid (CFU-GM) precursors in the nonadherent phase was determined. The morphology of the fetal liver nucleated cells during culture was also examined. This study shows that fetal liver stromas efficiently support fetal BFU-e for 6 to 7 weeks in vitro. Bone marrow stromas

were not able to maintain fetal BFU-e beyond 4 weeks. Significant numbers of marrow BFU-e were not sustained in vitro on either source of stroma. On the other hand, the stroma layers of fetal liver and marrow origin were equally effective in maintaining fetal CFU-GM and adult CFU-GM in long-term culture. These findings show that the human embryonic liver stroma is a preferential site for stimulating fetal erythropoiesis. They do not demonstrate differences in stroma function to explain the relative paucity of myelopoiesis in the fetal liver.

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FROM THE SIXTH until the 23rd week of gestation, the fetal liver is the main site of hematopoiesis of the human embryo.^{1,2} Although hematopoiesis is almost entirely erythroid, there is a high concentration of myeloid progenitor cells (CFU-GM) in early trimester human fetal livers, similar to that in adult bone marrow.¹ In midtrimester fetuses,² the organ continues to mainly show active erythropoiesis, despite the high frequency of CFU-GM in the tissue.

After regression of hematopoiesis in the liver, the stem cells migrate to the bone marrow, where hematopoiesis becomes established. The questions why erythropoiesis dominates in the fetal liver notwithstanding the large numbers of myeloid precursor cells and why at a certain time during ontogeny hematopoiesis settles in the bone marrow have not been clarified. These phenomena may be caused, for example, by alterations in the properties of the stem cells during prenatal development or by changes of the environmental properties of the stroma that sustain and govern hematopoietic stem cell differentiation. In order to throw light on these questions, we have performed long-term cultures according to Dexter et al.^{3,4}

Fetal liver and bone marrow-derived stroma layers were established and then seeded with fetal liver or bone marrow cells. The effects of the stroma layers, derived from either source, on the proliferation and differentiation of human fetal and adult marrow progenitor cells were assessed.

MATERIALS AND METHODS

Human fetal liver cells and bone marrow cells. Midtrimester fetuses (12 to 22 weeks) were obtained after vaginal termination of the pregnancy by the dilatation and evacuation method of Finks.⁵ Fetal age was estimated from the duration of amenorrhea and verified by echoscopy. The livers were collected in sterile Hanks' balanced salt solution (HBSS), supplemented with HEPES and antibiotics, and transported to the laboratory. Within one to three hours after collection, the livers were washed three times with HBSS, and a single-cell suspension was prepared by pressing the cells through a nylon filter. The cells were passed through an 0.8-mm needle and filtrated through a Jena glass filter (D2, 90 to 40 μ m). The nucleated cells were counted in Türk solution. The viability was estimated by trypan blue exclusion and varied between 65% and 85%.

Bone marrow cells were aspirated from the posterior iliac spine of adult volunteers who had no hematologic abnormalities. The nucleated marrow cells were separated after 0.1% methylcellulose

sedimentation. All patients had given informed consent to donate marrow in accordance with institutional guidelines.

Parts of the fetal liver and bone marrow cells were directly used for preparing adherent stroma layers in culture; the remainders of the cell suspensions were cryopreserved.

Long-term cultures. Bone marrow stroma layers, derived from 20×10^6 nucleated marrow cells per flask, were established during 2 to 3 weeks in 8 mL of alpha medium containing 20% horse serum and 10^{-7} mol/L hydrocortisone acetate as described by Touw and Löwenberg.⁶ Fetal liver adherent cell layers were established during 3 to 5 weeks after inoculation of 15×10^6 nucleated fetal liver cells into T flasks. After stroma formation, the fetal liver and bone marrow cells were thawed and separated on Ficoll Isopaque (1.077 g/mL). Then 5 to 10×10^6 of these nucleated cells were inoculated in cultures in which stroma layers had been preestablished. Cultures were run in duplicate. Various combinations of stroma layer and recharge were examined in comparative experiments: autologous fetal liver cells on a fetal liver stroma, allogeneic fetal liver cells on a fetal liver stroma, allogeneic fetal liver cells on a bone marrow stroma, and allogeneic bone marrow cells on a fetal liver stroma. To exclude the possibility that the progenitors arose from the adherent cell layer, in selected experiments the stroma layers were irradiated (25 Gy) before recharge. Control experiments were also performed by culturing fetal liver cells in the absence of a stroma layer. At weekly intervals the nonadherent cells, in the collected half-volume of the culture medium, were counted and assayed for myeloid and erythroid progenitor cell content (CFU-GM, BFU-e), and the medium was replaced by fresh medium. The number of progenitor cells are expressed as the total number per flask and the values are corrected for weekly half-volume depletion ($A \times 2^{n-1}$, A = number of recovered progenitor cells as determined in two flasks, n = number of weeks in culture). In selected experiments, the morphology of the cells was examined as well.

Colony-forming assays. Granulocyte-macrophage colonies were grown in a double layer semisolid agar system, supplemented with 20% to 30% human placental conditioned medium (HPCM) in

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the underlayer, as described,⁶ to determine the number of myeloid precursors (CFU-GM). Erythroid burst-forming units (BFU-e) were cultured in 1.1% methylcellulose in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Scotland), supplemented with crude human urinary erythropoietin (prepared from urine of patients with aplastic anemia), 30% fetal calf serum, bovine serum albumin (0.75%), egg lecithin (3×10^{-5} mol/L), Na_2SeO_3 (10^{-7} mol/L), iron-saturated human transferrin (7.7×10^{-6} mol/L), and beta-mercaptoethanol (5×10^{-5} mol/L). One milliliter of this culture medium, containing 1×10^5 marrow or fetal liver cells, was plated into four wells of a cluster dish (Costar, Cambridge, MA; 16-mm well diameter) and incubated at 37 °C in a fully humidified atmosphere with 5% CO_2 in air. Colonies containing more than 50 cells were counted on day 14 by use of an invertoscope (Zeiss).

The number of CFU-GM and BFU-e per 10^5 cells in the fetal liver preparations of varying embryonic ages at the time of collection were determined. Early and late midtrimester fetal livers contained approximately equivalent concentrations of precursor cells. Fetal age 13 to 15 weeks: CFU-GM = 694 ± 309 ($n = 26$) (mean $\pm$ SD), BFU-e = $3,917 \pm 2,420$ ($n = 14$); 16 to 18 weeks fetal age: CFU-GM = 754 ± 397 ($n = 34$), BFU-e = $4,166 \pm 2,009$ ($n = 20$).

Cell morphology. In order to follow the lineage-specific maturation during long-term culture, in some experiments the cells were used for cytological examination. Samples of the nonadherent cells were collected, cytocentrifuged (Shandon, Cheshire, England) and stained with May-Grünwald-Giemsa (Merck). Per differential count, 200 cells were scored. Fetal liver and bone marrow stromal layers were also examined after cytochemical staining with α -naphthyl-esterase (with and without sodium fluoride), alkaline phosphatase, and acid phosphatase.

RESULTS

The ability of fetal liver stroma layers to maintain fetal liver cells in Dexter-type cultures. In six experiments (Table 1) we recharged fetal liver-derived adherent cell

layers with cryopreserved autologous fetal liver cells, to determine the maintenance of CFU-GM and BFU-e in long-term culture. A typical decline of CFU-GM numbers was seen in all instances during the first week, which, in four experiments, was subsequently followed by a maintenance of CFU-GM during another 5 to 6 weeks. In four experiments CFU-GM numbers were still detectable at week 6 to 7 of culture, while in the two other experiments (2 and 6) CFU-GM extinguished rapidly. Numbers of BFU-e were also assessed throughout culture and were recovered until approximately week 6 of culture. BFU-e showed similar kinetics of recovery in vitro, ie, a precipitous fall during week 1 and a maintenance of BFU-e numbers during the subsequent 6 weeks of culture. The data are not indicative of a relationship between fetal age of the stromas and their capacity to support CFU-GM and BFU-e during culture. Stromas derived from livers of week 14 and week 15 fetuses (Table 1, experiments 1, 2, and 3) and those from week 18 (Table 1, experiments 4, 5, and 6) gave equivalent results.

In order to investigate the capacity of fetal liver stroma layers to sustain the fetal liver hematopoiesis from different donors, we also evaluated the effect of allogeneic stroma layers under similar conditions. Two directly comparative experiments were performed, and the influence of the allogeneic fetal liver stroma on the recoveries of nucleated cells, CFU-GM, and BFU-e in long-term cultures was determined. Equivalent values of these parameters were measured on allogeneic and syngeneic fetal liver stroma layers.

In addition, four control experiments were performed in which fetal liver cells were cultured in the absence of a stroma layer. In these cultures no CFU-GM and BFU-e could be recovered at week 4 of culture. In two experiments, the cell layers were scraped and assayed for the presence of

Table 1. CFU-GM and BFU-e in Stromal Cultures Determined at Weekly Intervals: Autologous Fetal Liver Cells on a Fetal Liver-Derived Adherent Cell Layer

	Experiment 1 (14)*	Experiment 2 (14)*	Experiment 3 (15)*	Experiment 4 (18)*	Experiment 5 (18)*	Experiment 6 (18)*
CFU-GM						
Day 0	40,000	14,300	14,896	9,724	12,096	38,300
Week 1	12,420	4,030	4,620	8,064	4,200	6,720
Week 2	3,220	224	112	386	600	1,098
Week 3	ND	120	1,120	920	200	4,180
Week 4	1,040	80	2,640	560	3,696	2,720
Week 5	1,120	0	320	640	4,352	0
Week 6	2,240		320	960	2,560	
Week 7	2,560		640	0	1,280	
BFU-e						
Day 0	174,000	114,000	106,780	60,665	73,500	218,500
Week 1	18,684	5,890	5,632	5,472	36,036	8,016
Week 2	6,552	4,480	2,072	3,050	7,008	2,530
Week 3	12,208	3,824	584	3,760	15,400	7,752
Week 4	4,664	2,032	992	2,936	22,088	4,840
Week 5	5,440	944	1,904	3,456	17,536	8,640
Week 6	10,208	704	864	1,728	22,112	3,456
Week 7	2,048	0	1,024	3,456	0	0

The upper part of the table presents the recoveries of CFU-GM numbers; the lower part, those of BFU-e numbers during long-term culture. Figures indicate corrected numbers of progenitors per flask. ND, not determined.

*Fetal age in weeks.

CFU-GM and BFU-e. This was done to verify whether significant numbers of progenitor cells were trapped in the adherent cell layer. At week 3 of culture, the fetal liver stroma layer contained 75 CFU-GM per flask and at the time of recharge (week 4) no CFU-GM were detected. At the same time points, no BFU-e were measurable in the fetal liver adherent cell layers.

Is the bone marrow stroma layer capable of maintaining allogeneic fetal liver cells in long-term culture? In a subsequent series of experiments, a comparison between the effects of bone marrow and fetal liver layers on fetal liver CFU-GM (Fig 1) and BFU-e (Fig 2) was made. These experiments were performed using allogeneic cells.

The myeloid progenitor cells from fetal liver were equally well maintained on fetal liver and bone marrow stroma layers until approximately week 9. No differences were seen as compared with cultures in which the stroma layers had been preirradiated, indicating that the CFU-GM measured in the nonadherent layer were not derived from the adherent layer. On the other hand, it appeared that bone marrow adherent cell layers were generally inferior to fetal liver stroma layers in maintaining fetal liver BFU-e numbers. This was apparent from the directly comparative experiments presented in Fig 2. Additional experiments revealed that considerable numbers of BFU-e were still recoverable at week 6 in five allogeneic fetal liver cultures (data not shown). However, when allogeneic fetal liver cells were cultured on a bone marrow stroma layer, BFU-e were measurable until 3 to 5 weeks only.

Thus these data indicate a stromal specificity of fetal liver and adult marrow for promoting the recoveries of the erythroid precursor cells but not myeloid precursor cells. Notably, preirradiation of the stroma layers did not influence the recoveries of the numbers of BFU-e from the cultures.

Is a fetal liver stroma layer also capable of maintaining

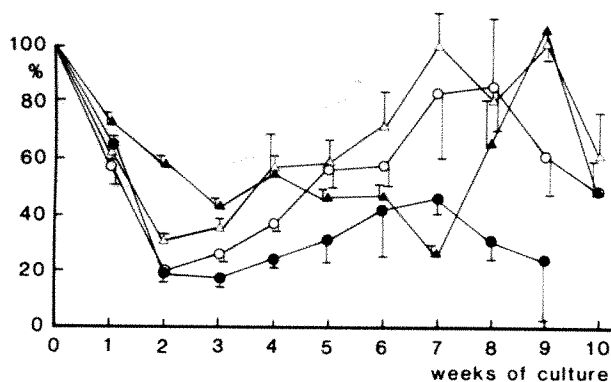


Fig 1. Direct comparison of the effect of fetal liver and bone marrow stromas on fetal liver CFU-GM. The curves represent percentage recoveries of the numbers of CFU-GM relative to those inoculated in culture at time 0 (100%) and are expressed as a function of the time (weeks) of culture. ▲—▲, fetal liver (autologous) in cultures with fetal liver stroma; ●—●, same but with preirradiated stroma; △—△, fetal liver (allogeneic) in culture with marrow stroma; ○—○, same but with preirradiated stroma. Fetal liver cells from the same source were inoculated on comparative stromal layers in duplicate (means $\pm$ SD).

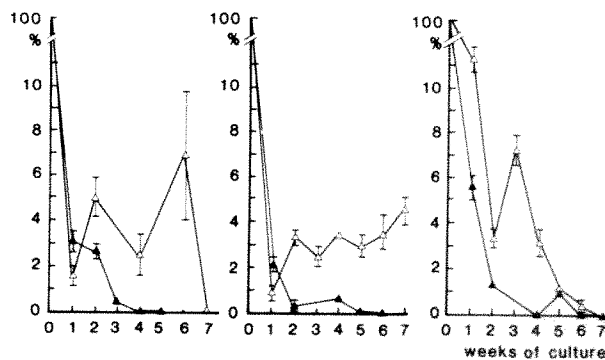


Fig 2. Comparison of the effect of fetal liver and bone marrow stromas on fetal liver BFU-e. The curves represent percentage recoveries of the numbers of BFU-e relative to those inoculated at time 0 (100%) and are expressed as a function of the time (weeks) of culture. Each section gives the experimental data (means $\pm$ SD) within individual experiments. △—△, cultures with fetal liver stroma; ▲—▲, cultures with marrow stroma.

adult precursor cells in long-term culture? In the last series of experiments, the capacity of fetal liver stromas to maintain marrow precursors (Table 2) in long-term cultures was examined. As compared with marrow stromas, fetal liver stromas were equally capable of maintaining marrow CFU-GM and BFU-e.

In conclusion, the favorable effect of a fetal liver stroma layer on the maintenance of erythroid progenitor cells is restricted to fetal liver BFU-e; it is not exerted on marrow BFU-e.

Morphology of the nonadherent fetal liver cells during long-term culture. The lineage-specific developments of the recharged fetal liver cells during long-term culture were assessed by cytological examination. Whereas the fetal liver is mainly erythropoietic at the time of inoculation, at 1 week of cultivation on a stroma layer, a switch to myelopoiesis was observed. After 4 to 5 weeks in vitro the nonadherent cell population was mainly composed of monocytes and macrophages. No differences were seen between cells grown on an autologous or allogeneic fetal liver stroma or those on a bone marrow stroma.

Morphology of the fetal liver stromal layers. The composition of the fetal liver stroma differed from that of the bone marrow stroma. The fetal liver fibroblasts were organized as confluent layers on the bottom of the flask. In contrast to the bone marrow cultures no patches or cobblestones were observed in cross sections of the fetal liver stroma. No fat cells appeared in the fetal liver stromal cultures as occurred in the bone marrow stromal cultures.⁶ Hematopoietic cells were scattered throughout the stroma layer. Cytochemistry of these cells did not reveal differences and confirmed the presence of macrophages, myeloblasts, and more mature myelocytic cells.

DISCUSSION

We have performed experiments to examine the specific effects of fetal liver and bone marrow stroma layers on the proliferation and differentiation of fetal and adult hemo-

Table 2. Comparison Between the Abilities of Marrow and Fetal Liver Stromas to Maintain Marrow Progenitors (CFU-GM; BFU-e)

	CFU-GM				BFU-E								
	Autologous BM Cells on a BM Stroma Layer	Allogeneic BM Cells on a Fetal Liver Stroma Layer			Autologous BM Cells on a BM Stroma Layer				Allogeneic BM Cells on a Fetal Liver Stroma Layer				
		Exp. 10	Exp. 11	Exp. 12	Exp. 13	Exp. 14	Exp. 15	Exp. 16	Exp. 17	Exp. 18	Exp. 19	Exp. 20	Exp. 21
Day 0	3,154	11,826	10,296	2,200	13,248	27,500	4,067	14,490	62,600	13,248	18,615	20,124	16,100
Week 1	2,106	4,258	2,460	1,270	26	1,973	855	13	24,249	320	2,438	1,770	4,109
Week 2	3,194	4,675	3,116	1,760	84	0	168	0	3,060	660	66	0	129
Week 3	2,840	456	616	1,440	0		0		0	240	0		0
Week 4	1,280	560	720	640						920			
Week 5	960	320	1,920	1,920									
Week 6	320	0	1,280	0									

Experiments 14 and 19 were done as direct comparisons with the same marrow specimen.

poietic precursors. The data presented in this study show that human fetal liver hematopoiesis in long-term culture, similar to human marrow hematopoiesis⁷⁻⁹ requires the establishment of an adherent cell layer. In direct comparative experiments it was demonstrated that the fetal liver stroma maintains fetal liver BFU-e better than the bone marrow stroma does. In contrast, marrow BFU-e, marrow CFU-GM, and fetal liver CFU-GM were supported equally well by the stroma layers of both tissues.¹⁰⁻¹² Allogeneic effects between stromas and cell inoculates were not apparent.

The hematopoietic inductive microenvironmental sites within different organs are thought to have a role in regulating hematopoiesis from the pluripotent stem cells into committed precursors.^{13,14} The microenvironments in different organs (eg, marrow, spleen, embryo liver) appear to have different specificities in determining cell lineage maturation.¹⁵ We investigated whether fetal liver stroma layers were capable of maintaining BFU-e in long-term cultures. The answer to this question is considered of interest with respect to the lack of understanding of the predominantly erythropoietic nature of the human fetal liver. Direct comparisons between the effects of fetal liver and marrow stroma layers on fetal BFU-e demonstrated a more powerful stimulative capacity of the fetal microenvironment.

BFU-e were recovered for 6 to 7 weeks when seeded on a fetal stroma, whereas, in all instances, they had disappeared before 5 weeks after their inoculation into marrow stromal cultures. Total BFU-e numbers recovered were also higher in fetal liver than in marrow stromal cultures. It is unclear which cell types in the stroma layers account for these differences. Evidence exists to suggest that macrophages and endothelial cells in human marrow stromal cultures do not influence the proliferation of marrow BFU-e.¹⁶ Marrow-derived fibroblasts appear to suppress the formation of (new) BFU-e. These cells may therefore be regarded as candidates being responsible for the discrepant effects of fetal liver and marrow stromas on BFU-e. Both in fetal liver and marrow stromal cultures BFU-e numbers dropped considerably within the first week, but in the case of fetal liver, a production of erythroid precursors was seen beyond week 2. We noted that the stimulative effect of the fetal liver microenvironment on erythroid progenitors was specific for

fetal BFU-e. When bone marrow cells were cultured on a fetal liver stroma layer, the number of adult BFU-e declined precipitously. These different values of fetal liver and marrow BFU-e on the same type of stroma are suggestive of cell dissimilarities existing between fetal liver BFU-e and marrow BFU-e and indicate alterations of the responsiveness of the erythroid precursors to stroma stimuli during human ontogeny.

Human fetal liver contains large numbers of committed myeloid progenitor cells. However, a minimal representation of granulopoiesis is apparent in histologic sections. Whether the scarce granulopoietic activity in the fetal liver organ is the result of different microenvironmental influences or instead a different responsiveness of the fetal progenitors to stimulation has not been defined. Our experiments do not reveal divergent efficiencies of fetal liver and marrow stromas as far as the production of CFU-GM is concerned. Quantitatively equivalent numbers of marrow- and fetal liver-derived CFU-GM were recovered from the fetal liver stromas (cultured for 5 to 7 weeks). In addition, fetal liver and marrow stroma layers permitted identical recoveries of CFU-GM numbers. Thus the data from these studies suggest that (a) fetal CFU-GM are susceptible to the stromal factors from both marrow and fetal liver, and (b) the fetal liver stroma layer is capable of stimulating the production of CFU-GM from adult and embryonic hematopoiesis to the same extent as bone marrow stroma layers do.

Morphological analysis revealed the predominance of myeloid cells, while erythroid cells had disappeared within 1 week of culture. As a matter of fact, it is likely that in vivo in the embryo, certain events inhibit granulopoiesis from the numerous myeloid precursors contained in the human fetal liver. A previous study¹⁰ has shown that mature granulopoietic elements from fetal liver may be detected in cultures on a bone marrow stroma layer for more than a year. This particular analysis did not include measurements of CFU-GM. We discontinued our cultures as soon as the CFU-GM had disappeared, and in our experience, at approximately week 5 of culture, the nonadherent cell population consisted mainly of macrophages, regardless of the tissue origin of the stroma layer or recharge.

We conclude from these experiments that with regard to

the regulation of erythropoiesis, both the function of the fetal liver microenvironment and the responsiveness of fetal liver progenitors (BFU-e) to stroma stimulation differ from those of the marrow stroma and marrow BFU-e. No differences were found to account for the scarcity of myelopoiesis in the fetal liver. While in vitro no active suppression of granulopoiesis exists, the rare granulopoiesis in vivo may primarily

be the consequence of the erythropoietic advantage in the embryo liver.

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Factor IX_{Alabama}: A Point Mutation in a Clotting Protein Results in Hemophilia B

By Lisa M. Davis, Royal A. McGraw, Jerry L. Ware, Harold R. Roberts, and Darrel W. Stafford

Factor IX_{Alabama} is a variant factor IX molecule responsible for a clinically moderate form of hemophilia B. Twenty-five kilobases (kb) of the variant gene, including seven exons coding for the structural protein, were cloned and characterized. The restriction map and the arrangement of coding regions are identical to those of the normal gene. DNA sequence analysis of the coding regions revealed a single base-pair difference between the gene for factor IX_{Alabama}

and the normal factor IX gene. An adenine to guanine transition in the first nucleotide of exon d causes the substitution of a glycine codon (GGT) for the normal aspartic acid codon (GAT). This point mutation results in a single amino acid substitution at residue 47 of the zymogen and represents the genetic defect in factor IX_{Alabama}.
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FACTOR IX is a plasma coagulation glycoprotein necessary for normal hemostasis. The factor IX zymogen has a mol wt of 56,000 daltons and can be activated by factor XIa and Ca²⁺ as well as factor VIIa-tissue factor, both of which cleave the molecule at arginyl residues 145 and 180 to release an activation peptide of ~10,000 daltons. Activated factor IX, in the presence of co-factors (Ca²⁺, phospholipid, and activated factor VIII) rapidly converts factor X to factor Xa.¹

The sequence of normal human factor IX cDNA clones and genomic clones has been previously reported.²⁻⁶ The normal gene is 35 kilobases (kb) in length and consists of eight coding regions (exons) and seven intervening sequences (introns). The full-length cDNA is 2.8 kb and includes 1.3 kb of a 3' nontranslated region. The gene has been localized to band Xq27 through Xq28 on the long arm of the X chromosome and is in close linkage with the fragile X mental retardation syndrome.⁷⁻⁹

Factor IX is absent or defective in the plasma of individuals suffering from hemophilia B (Christmas disease). This sex-linked bleeding disorder results from a variety of genetic defects in the factor IX gene.¹ Affected individuals are classified according to their plasma levels of immunologically detectable factor IX. Cross-reacting material-positive (CRM⁺) variants have essentially normal levels of factor IX antigen with reduced clotting activity. CRM-reduced (CRM^{-R}) patients have proportionately reduced levels of both antigen and clotting activity and CRM-negative (CRM⁻) individuals have virtually undetectable levels of both antigen and clotting activity.^{1,10,11}

At least eight unique CRM⁺ variants have been described.¹ The CRM⁺ variants provide a unique opportunity for understanding the complex relationship between the

protein structure of factor IX and its functional role in coagulation. When a specific mutation in one of these variants is identified and correlated with an aberrant function, an understanding of the relationship between structure and function is enhanced. One CRM⁺ variant, factor IX_{Alabama}, has been isolated from patients with moderate hemophilia B. Factor IX_{Alabama} has only 10% of the clotting activity of normal factor IX.^{12,13} In this report, we describe the genetic defect in patients with factor IX_{Alabama}. Our studies reveal a point mutation resulting in the substitution of a glycine for an aspartic acid at position 47 of the factor IX protein.

MATERIALS AND METHODS

Patients. Patients from the pedigree with factor IX_{Alabama} have been previously described.^{12,13} Factor IX was purified from the plasma of affected patients.¹⁴ After informed consent from patients, DNA was extracted from whole blood or leukocytes from the two affected patients in this kindred list.¹⁵

Factor IX_{Alabama}. Factor IX_{Alabama} was isolated from the patients' plasma as previously described.¹⁶ The mol wt and determination of γ -carboxyglutamic acid residues were performed essentially as described for factor IX_{Chapel Hill}.¹⁶ β -hydroxyaspartic acid was determined by Dr Walter Kiesel of the University of Washington, Seattle.¹⁷

Factor IX_{Alabama} genomic library. The recombinant library was constructed by ligating a size-fractionated *Eco*RI partial digest of this genomic DNA to the phage vector λ gtl- λ B.¹⁸ Conditions for sucrose gradient fractionation, ligation, and λ packaging were as described.¹⁹

Factor IX_{Alabama} clones. The library was screened for factor IX_{Alabama} clones with synthetic oligonucleotide probes or with the full-length factor IX cDNA.⁴ Oligonucleotides were end-labeled as described,¹⁹ and the full-length cDNA was radiolabeled according to a standard nick-translation protocol.²⁰ Alternatively, the full-length cDNA, subcloned into the M13 phage vector mp9, was labeled with α -³²P dATP in a reaction similar to the chain-terminating dideoxy DNA-sequencing reaction except that dideoxy nucleotides were omitted.

The factor IX_{Alabama} clones were subcloned from the λ phage vector into both pUC plasmids and M13 phage vectors to facilitate restriction analysis and DNA sequence determination. Standard procedures for construction of these subclones were followed.²¹⁻²³

DNA sequence analysis. DNA sequence analysis was done according to the method of Biggin and co-workers²⁴ or by a modification of the Sanger dideoxy chain-terminating method²⁵ using end-labeled primers.²⁶ Primer was either the universal pentadecamer (New England Biolabs) or one of the factor IX-specific synthetic oligonucleotides.⁴ The template was either an M13 single-stranded DNA or a pUC double-stranded DNA subclone.

RESULTS

The mol wt of factor IX_{Alabama} was normal. The protein has 12 γ carboxyglutamic acid residues and a β -hydroxy-

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aspartic acid at position 64 (data not shown). Factor IX can be activated to the factor IXa β form of the molecule and possesses 10% of the clotting activity of normal factor IXa β . In addition, factor IX_{Alabama} is present in normal amounts, as previously demonstrated.¹²

Two factor IX genomic clones, designated LC-7 and LC-10, were isolated from the factor IX_{Alabama} library. Together they comprise 25 kb of the factor IX_{Alabama} gene. These clones include all the sequence coding for the mature structural protein and one-third of the leader peptide. The remainder of the pre-pro sequence was not determined, since the factor IX_{Alabama} protein was secreted normally, as evidenced by normal plasma antigen concentrations, and had a normal complement of γ carboxyglutamic acid residues. These findings indicated that the pre-pro portion of the gene was normal.

LC-7 was identified with an end-labeled 18-mer oligonucleotide probe, synthesized for the active serine site region of exon h. LC-10 was identified with the uniformly labeled, Klenow-extended, full-length cDNA. The identity of each clone was established initially as follows: a *Sau* 3A digest of each clone was subcloned into the *Bam*HI site of mp8 and rescreened with the original probe. The DNA sequences of positive plaques were determined and compared with the published cDNA sequences.²⁻⁴

DNA sequence analysis. Figure 1 shows the strategy used in sequencing factor IX_{Alabama}. In those cases in which sequence of only one strand was obtained, several independently derived templates were used. Because exon d contains the point substitution found to cause the functional defect of

factor IX_{Alabama} (discussed below), the DNA sequence of this region was determined in both strands from six independent M13 templates derived from different λ bacteriophage preparations.

The nucleotide sequence for ~4,600 bases of the factor IX_{Alabama} gene was determined. This included all the sequence coding for the mature structural protein, one-third of the leader peptide sequence, all the 3' nontranslated region, and some flanking intron sequence (Table 1).

The sequence throughout the mature coding region of factor IX_{Alabama} is identical to all the normal factor IX clones except at two positions. Both affect the primary amino acid sequence. One difference occurs in the third residue of the activation peptide and has been shown by us to result from a nondeleterious dimorphism that is found in both the normal and hemophilic population.⁴ The presence of either a guanine or an adenine in the first position of the codon for the third residue of the activation peptide results in either an alanine (GCT) or threonine (ACT) in this position. This corresponds to residue 148 of the zymogen. Factor IX_{Alabama} codes for a threonine in this position.

The other difference affecting the amino acid sequence is not found in any of the normal factor IX sequences and constitutes the abnormality in factor IX_{Alabama}. This difference occurs in the first nucleotide of exon d (Fig 2A). This is the second nucleotide of the triplet coding for the 47th residue of the mature protein. An adenine to guanine transition causes the substitution of a GGT codon for the normal GAT codon. This mutation results in a glycine substitution for aspartic acid at residue 47, preceding the epidermal growth-factorlike region of the protein. Figure 2B shows more clearly the DNA coding sequence of normal factor IX and factor IX_{Alabama} in this region and the corresponding translated amino acid sequence.

Two genomic factor IX sequences^{5,6} are available for comparison of intron sequences that demonstrate naturally occurring polymorphisms. Anson and colleagues⁵ reported the presence of a thymine between the cytosine and adenine that occur at 124 and 125 nucleotides 5' to exon b. This thymine is absent in both factor IX_{Alabama} and in the normal sequence, as reported by Yoshitake and co-workers.⁶ The sequence of one cDNA clone⁴ and two genomic clones^{5,6} that include the complete 3' nontranslated region have been reported. There are discrepancies between those sequences

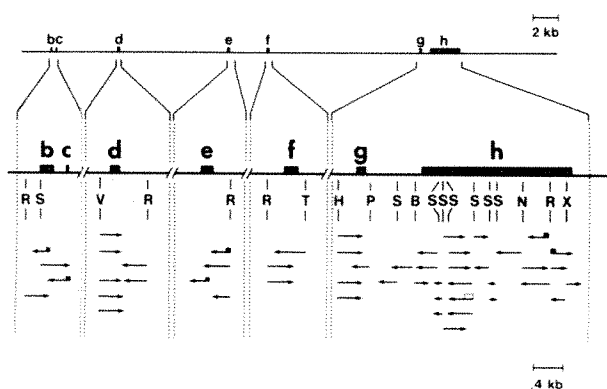


Fig 1. Sequencing strategy. The gene is shown at top, with exons b through h enlarged in the middle. Vertical lines represent termini of restriction fragments subcloned for DNA sequence analysis: R: *Eco*RI, S: *Sau* 3A, V: *Pvu*II, T: *Sst*I, H: *Hind*III, P: *Pst*I, B: *Bgl*II, N: *Nco*I, X: *Xba*I. Each arrow represents the extent and direction of sequence obtained from individual template and primer combinations. Arrows beginning with solid boxes indicate sequence obtained from double-stranded DNA templates; simple arrows indicate sequence determined from M13 single-stranded DNA templates, using the commercial universal primer. The single arrow beginning with an open box represents sequence obtained from an M13 single-stranded DNA template, using the synthetic 18-mer as primer. The five arrows showing determination of exon d sequence were the result of two independent ligations of a 0.76-kilobase *Eco*RI/*Pvu*II fragment. Additional sequence analysis confirmed the exon d mutation in both strands (additional arrow not shown).

Table 1. Summary of Determined FIX_{Alabama} Nucleotide Sequence

Exon	Exon Length	Nucleotides 5' to Exon	Nucleotides 3' to Exon
b	164	190	188
c	25	188*	9
d	114	106	140
e	129	106	197
f	203	226	35
g	115	228	40
h	1,963†	394	54
Totals	2,713	1,250	663

*This sequence is also listed as nucleotides 3' to exon b and has not been included in the total.

†Includes 1,418 nucleotides at the 3' end that are not translated.

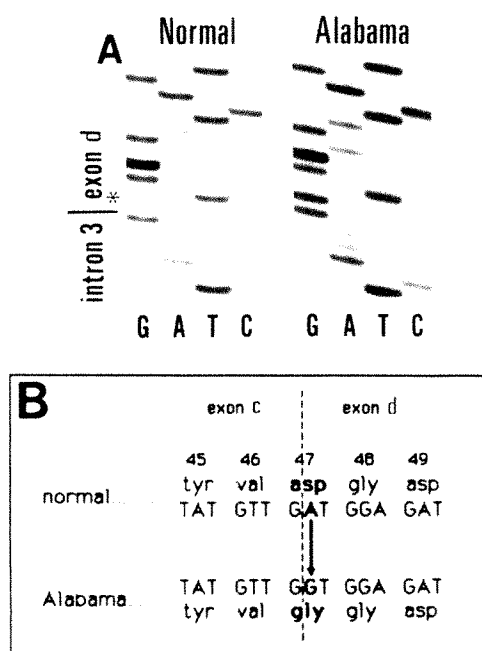


Fig 2. (A) Sequence analysis of factor IX_{Alabama}. Sequencing data is shown for the region of junction between intron 3 and exon d. The first nucleotide of exon d in the normal sequence (left autoradiograph) is adenine as compared with the guanine substitution in factor IX_{Alabama} (right autoradiograph). Both normal and mutant nucleotides are highlighted at the level of the asterisk. (B) The abnormal amino acid sequence in factor IX_{Alabama}. The cDNA and amino acid sequence of normal factor IX are shown at top. The cDNA and amino acid sequence of factor IX_{Alabama} are shown at bottom. Substitution of guanine for adenine in the second nucleotide of the 47th codon predicts that glycine will be substituted for the normal aspartic acid.

and the one in this report at four positions, all involving single base-pair changes. When the sequence numbers of Yoshitake and co-workers are used as a reference,⁶ the cDNA sequence and the genomic sequence show cytosine at 31,663 where factor IX_{Alabama} has thymine; similarly, both the cDNA clone and the genomic clones have adenine at position 32,056 where factor IX_{Alabama} has guanine. Factor IX_{Alabama}, however, our cDNA clone,⁴ and the normal genomic sequence⁶ are alike at two additional positions, (adenine at 32,063 and cytosine at 32,308) but different from the cDNA sequence reported by Anson and colleagues,⁵ which has guanine at both positions.

DISCUSSION

Factor IX_{Alabama} is a dysfunctional coagulation protein with a normal mol wt and a normal complement of γ carboxyglutamic acid residues,¹ suggesting that the defect is not due to gross structural alterations of the gene. Our results obtained by both restriction and DNA sequence analysis of the clones confirm that the gross structure of the factor IX gene is intact. DNA sequence analysis, however, reveals a single base-pair substitution in the coding region of factor IX_{Alabama}; an adenine to guanine transition at nucleotide 307 in the cDNA sequence⁵ (corresponds to nucleotide 10,392 in

ref 6) changes a GAT codon to a GGT codon, resulting in a glycine substitution for aspartic acid at position 47 of the protein. The substitution occurs in the amino terminal region of the "connecting peptide" in the light chain of factor IX preceding the epidermal growth factor domain. This single point substitution is the only observed structural difference between factor IX_{Alabama} and normal factor IX, suggesting that this is the genetic defect responsible for the decreased clotting activity of factor IX_{Alabama}. This substitution is sufficient to result in moderate bleeding in affected members of the factor IX_{Alabama} kindred.

DNA sequence of exon a, which codes for two-thirds of the leader peptide, was not determined. The leader peptide is believed to function in transport of the protein from the site of synthesis to its site of carboxylation in the cytoplasm of the hematocyte before secretion into the circulation. Because factor IX_{Alabama} is normally secreted and carboxylated, it is very unlikely that a defect exists in the region of exon a. Therefore absence of the sequence of exon a should not affect the conclusions drawn here regarding the defect responsible for the bleeding tendency in affected patients with factor IX_{Alabama}.

The DNA sequence of exon d was confirmed in both strands. The possibility that the substitution might be a cloning artifact is diminished by the fact that the sequence was determined from six independent templates derived from two independent preparations of the λ clone LC-10. In addition, primary amino acid sequence analysis of the isolated factor IX_{Alabama} protein confirms the presence of glycine instead of aspartic acid at position 47.²⁷

Six other nucleotide differences exist between this variant gene and previously published sequences of normal factor IX genes, but five of these do not affect the structural protein and cannot account for the defect in factor IX_{Alabama}. The sixth difference is a naturally occurring dimorphism in the third residue of the activation peptide⁴ and again cannot be responsible for the dysfunctional protein because the dimorphism is observed in both normal factor IX and factor IX_{Alabama} and in other abnormal factor IX molecules.

Together, these facts suggest that a glycine substitution for aspartic acid at residue 47 is responsible for the decreased clotting activity of the factor IX_{Alabama} molecule. Aspartic acid is a relatively large, charged residue, usually found on the surface of globular proteins, where it may interact with other proteins or cofactors. Substitution of the small, uncharged glycine may interfere with normal charge interactions occurring in this region of the protein. Alternatively, the substitution may lead to an altered conformation by virtue of the relatively flexible C-N bond of glycine. Whether the functional defect of factor IX_{Alabama} results primarily from the loss of charge interactions, an altered conformation, or some other function, remains to be established. Previous work from our laboratory has shown, however, that the binding of activated factor IX_{Alabama} with phospholipid is normal, although clotting activity is abnormal.²⁸ Thus, we assume that the functional abnormality causing the decreased clotting activity of factor IX_{Alabama} is due to the mutation at position 47 of the zymogen, although the mechanism of the defect is not yet clear and awaits further study.

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Inhibition of Hemoglobin Production by Transferrin-Gallium

By Christopher R. Chitambar and Zorica Zivkovic

Recent clinical trials evaluating gallium nitrate as a chemotherapeutic agent have reported the development of microcytic hypochromic anemia in patients treated with this agent. Because gallium is known to bind avidly to transferrin, we examined the effect of transferrin-gallium (Tf-Ga) on hemoglobin production by Friend erythroleukemia cells in vitro. Cellular hemoglobin production, as assessed by benzidine staining, cellular hemoglobin content, and ^{59}Fe incorporation into heme, was significantly decreased following exposure of cells to Tf-Ga. Tf-Ga led to an early decrease in cellular ^{59}Fe incorporation even before changes in hemoglobin production were detected. A marked increase in cellular transferrin receptor expression

occurred following exposure of cells to Tf-Ga. Tf-Ga inhibition of hemoglobin production could be reversed and hemoglobin production could be restored to normal by addition to the media of either transferrin-iron (Tf-Fe) or iron-pyridoxal isonicotinoyl hydrazone, a compound capable of supplying iron directly to reticulocytes for heme synthesis without transferrin as a mediator. These studies provide an explanation for the development of anemia in patients treated with gallium nitrate and suggest that gallium's mechanism of chemotherapeutic action includes inhibition of cellular iron incorporation.

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GALLIUM, a group IIIa metal with significant antitumor activity,^{1,2} is currently being evaluated as a cancer chemotherapeutic agent in phase II clinical trials (gallium nitrate NSC-1500). A recent clinical study reported the development of microcytic hypochromic anemia in patients treated with this agent.² Although most chemotherapeutic drugs are myelosuppressive, the resultant anemia is usually normocytic normochromic, and the development of a microcytic hypochromic anemia from chemotherapy per se is unusual.

Several studies have demonstrated that gallium shares certain characteristics with iron in that it avidly binds to transferrin³ and lactoferrin^{4,5} and can be incorporated into ferritin.⁶ The uptake of gallium-67 by cells in tissue culture is markedly enhanced by the addition of exogenous transferrin to the media^{7,8} and, although the process of gallium uptake by cells is incompletely understood, it may be mediated by the transferrin receptor.⁹ These similarities between the transport and cellular uptake of iron and gallium raise the question of whether one metal might influence the cellular handling of the other and whether such an interaction could explain the development of hypochromic microcytic anemia during treatment with gallium nitrate.

To understand the mechanism of gallium-induced anemia better, we studied the effects of transferrin-gallium (Tf-Ga) on hemoglobin production by Friend erythroleukemia cells in vitro during induction with dimethylsulfoxide (DMSO). Hemoglobin production by these cells is significantly inhibited by Tf-Ga and this decrease in hemoglobin production is

preceded by a decrease in cellular iron incorporation. Provision of adequate amounts of iron in the form of transferrin-iron (Tf-Fe) or iron-pyridoxal isonicotinoyl hydrazone (Fe-PIH)¹⁰ reverses the Tf-Ga inhibition of hemoglobin production, indicating that the anemia seen in patients during gallium nitrate treatment is due specifically to inhibition of iron incorporation into developing erythroid precursors.

MATERIALS AND METHODS

Human transferrin, ferric chloride, ferric citrate, pyridoxal hydrochloride, isonicotinic acid hydrazide, benzidine dihydrochloride, Triton X-100, and DMSO were purchased from Sigma Chemical (St Louis). Gallium nitrate was obtained from Alfa Products (Danvers, MA), and cytosine arabinoside (Ara-C) was obtained from Upjohn (Kalamazoo, MI). ^{125}I -sodium iodide and $^{59}\text{FeCl}_3$ were obtained from New England Nuclear (Boston) and Amersham (Arlington Heights, IL), respectively. Saturation of apotransferrin with ^{59}Fe and iodination of ^{59}Fe -transferrin were performed as previously described.¹¹

Tf-Fe and Tf-Ga were prepared by using modifications of previously described methods.¹² To ensure saturation of transferrin, 3 mol of metal (as ferric chloride or gallium nitrate) was added to each mol of apotransferrin (apoTf) dissolved in 20 mmol/L of acetic acid and 150 mmol/L of NaCl pH 3.5. The pH of this solution was slowly raised to 7.4 with 1 mol/L of NaHCO_3 , and the saturation of transferrin by gallium or iron was confirmed spectrophotometrically by measuring the change in absorbance at 242 nm for Tf-Ga³ and 465 nm for Tf-Fe.¹³ Stock solutions (10 mg protein/mL) of the two transferrin forms were thus prepared and used for subsequent experiments.

PIH and ferric-PIH (Fe-PIH) were prepared according to the method of Ponka and co-workers.¹⁴ Equimolar solutions of isonicotinic acid hydrazide and pyridoxal HCl in sodium acetate buffer were heated in a boiling water bath for five minutes, and the PIH precipitate that formed on cooling was washed, filtered, and dried. Fe-PIH was prepared by combining PIH with a solution of ferric citrate in a ratio of 2 moles of PIH:1 mole Fe^{3+} . The yellow PIH precipitate immediately turned red-brown when mixed with ferric citrate. The insoluble Fe-PIH complex was thoroughly washed, filtered, and dried. Fe-PIH, thus prepared, was dissolved by resuspending it in 150 mmol/L of NaCl, 10 mmol/L of KPO_4 pH 7.4 buffer, and by lowering the pH with a small amount of 0.15 mol/L of HCl. A 1-mmol/L stock solution of Fe-PIH was prepared so that the addition of Fe-PIH to the media to achieve final concentrations of 5 to 100 $\mu\text{mol/L}$ did not alter the pH of the tissue culture media.

Tissue culture. Friend erythroleukemia cells generously provided by Drs Asterios Tsiftoglou and Stephen Robinson were maintained in RPMI 1640 media containing 10% fetal calf serum

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(FCS) with 200 $\mu\text{g}/\text{mL}$ of penicillin and 0.2 mg/mL of streptomycin (regular media). The original cells were further subcloned to yield a clone in which 80% to 90% of the cells demonstrated hemoglobin production after three days of exposure to 2% DMSO. The percentage of hemoglobin-producing cells was determined by benzidine staining,¹⁵ and the amount of hemoglobin produced was measured according to the method of Tsiftoglou and colleagues¹⁶ using bovine hemoglobin as a standard. In experiments examining hemoglobin production, cells were plated at 1×10^5 cells/mL in regular media or media containing 2% DMSO with various concentrations of apo-transferrin, Tf-Fe, Tf-Ga, or Ara-C. Aliquots of cells were harvested at different times following initial plating, and the effects of the different media additives on hemoglobin production were determined. In coinubation studies of hemoglobin production, cells plated in media containing 2% DMSO and Tf-Ga were also exposed to various concentrations of Tf-Fe (100 to 400 $\mu\text{g}/\text{mL}$) or Fe-PIH. In other experiments, cells were removed from media containing Tf-Ga after 24, 48, or 72 hours and were washed and resuspended at their original density in fresh media containing 2% DMSO without Tf-Ga. In converse experiments, Tf-Ga was added to the media after cells had been exposed to 2% DMSO for 24, 48, or 72 hours, and hemoglobin production was assessed by benzidine staining.

Cellular uptake of ^{59}Fe . ^{59}Fe -Tf (2.5 $\text{ng Fe}/\text{mL}$) was added to cells plated at time 0 hour in media containing 2% DMSO plus 100, 200, or 400 $\mu\text{g}/\text{mL}$ of apoTf or Tf-Ga. Following an incubation of 24 or 72 hours, cells were harvested, counted, and washed by centrifugation. The cell pellets were counted for radioactivity, and the uptake of $^{59}\text{Fe}/10^6$ cells was determined.

Intracellular distribution of ^{59}Fe following induction of hemoglobin production. Following 66 hours of cell growth in regular media or in media containing 2% DMSO with or without Tf-Ga, aliquots containing similar numbers of cells were harvested, washed with cold serum-free media and resuspended in 10 mL of serum-free media in T25- cm^2 tissue culture flasks (3×10^6 cells/mL per flask). ^{59}Fe -Tf (3.6 $\text{ng Fe}/\text{mL}$) was added to each flask, and the cells were incubated at 37 $^\circ\text{C}$ for six hours. Following this, the cells were harvested, washed with 10 mmol/L of Tris and 150 mmol/L of NaCl pH 7.4, and finally were resuspended in 1 mL of the same buffer containing 0.1% Triton X-100. The cells were disrupted by sonication and incubated overnight at 4 $^\circ\text{C}$. Cellular debris was removed by centrifugation at 10,000 g, and the cytoplasmic supernatant (containing 85% of the total ^{59}Fe radioactivity) was applied to a Sephadex G150 $1.5 \times 30\text{-cm}$ column equilibrated with the same buffer; 2-mL fractions were collected and counted for radioactivity.

^{125}I -Tf binding studies. Aliquots of cells growing in regular media, 2% DMSO media alone, or 2% DMSO media containing 200 or 400 $\mu\text{g}/\text{mL}$ of Tf-Ga were harvested at 24, 48, 72, and 96 hours following initial plating. The cells were washed twice in 10 mmol/L of KPO_4 , 150 mmol/L of NaCl pH 7.4 buffer containing 0.1% phosphate-buffered saline-bovine serum albumin (PBS-BSA), and the cell pellets were resuspended in 15 mL of PBS-BSA and incubated at 37 $^\circ\text{C}$ for 30 minutes. This 37 $^\circ\text{C}$ "preincubation" step was performed to deplete the cells of any receptor-bound transferrin and to make available all transferrin binding sites for the ^{125}I -Tf binding assay. Using immunoassays for transferrin described elsewhere,¹⁷ we have found that >95% of bound transferrin is removed with this preincubation step. Cells were then cooled to 4 $^\circ\text{C}$, washed with ice-cold PBS-BSA, and resuspended in 2 to 3 mL of the same buffer. ^{125}I -Tf binding to intact cells was performed at 4 $^\circ\text{C}$ as previously described,^{11,12} and maximal transferrin binding was calculated according to the method of Scatchard.¹⁸

RESULTS

Cell growth and cellular hemoglobin production. The addition of Tf-Ga at concentrations of 200 and 400 $\mu\text{g}/\text{mL}$ to

cells plated in media containing 2% DMSO resulted in a slight to moderate dose-dependent decrease in the rate of proliferation (Fig 1). Cells exposed to 400 $\mu\text{g}/\text{mL}$ of Tf-Ga reached a maximum density after 72 hours of incubation, with a subsequent small decrease in number by 96 hours. Cells exposed to 200 $\mu\text{g}/\text{mL}$ of Tf-Ga continued to increase in number, reaching a maximum density by 96 hours (Fig 1).

Cells exposed to 2% DMSO rapidly synthesized hemoglobin, as assessed by benzidine staining for hemoglobin-producing cells as well as by measurements of cellular hemoglobin content. In this particular subclone of Friend erythroleukemia cells, maximum benzidine positivity occurred after 72 hours of exposure to 2% DMSO, with no further increase in benzidine-positive cells at 96 hours (Fig 2). The addition of 100 to 500 $\mu\text{g}/\text{mL}$ of Tf-Fe or apoTf or 100 $\mu\text{g}/\text{mL}$ of Tf-Ga did not alter the percentage of hemoglobin-producing cells. With the addition of Tf-Ga (200 and 400 $\mu\text{g}/\text{mL}$) to the media, however, a significant decrease in hemoglobin-producing cells occurred (Fig 2). Like the control cells, cells exposed to Tf-Ga also achieved their maximum number of hemoglobin-positive cells by 72 hours, with a slight decrease in benzidine positivity thereafter. The hemoglobin content at 72 hours was $17.15 \pm 2.15 \mu\text{g}/10^7$ cells for control cells, whereas the hemoglobin content of cells exposed to 200 and 400 $\mu\text{g}/\text{mL}$ of Tf-Ga was $3.94 \pm 0.94 \mu\text{g}/10^7$ cells and $1.1 \pm 0.27 \mu\text{g}/10^7$ cells, respectively (mean $\pm$ SD of three experiments). Decreases in cellular hemoglobin production by Tf-Ga could also be demonstrated by studying the relative incorporation of ^{59}Fe into ferritin and hemoglobin during hemoglobin synthesis. Separation of subcellular components by Sephadex column chromatography revealed that ~75% of the intracellular ^{59}Fe was associated with hemoglobin in the control cells. In contrast, Tf-Ga-treated cells contained a much smaller amount of ^{59}Fe in the hemoglobin fraction, consistent with the decrease in hemoglobin production by these cells. Because maximum benzidine positivity was reached after 72 hours of incubation and

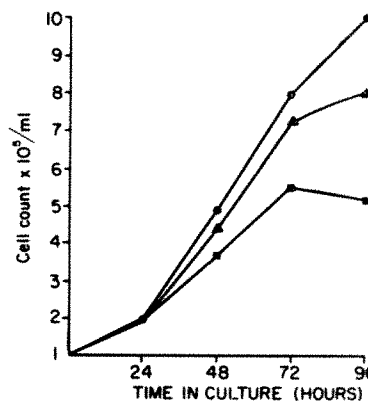


Fig 1. Effect of transferrin-gallium (Tf-Ga) on proliferation of Friend erythroleukemia cells. Friend erythroleukemia cells were plated at 1×10^5 cells/mL in media containing 2% dimethylsulfoxide (DMSO) plus different transferrin-forms. No additives, 100 $\mu\text{g}/\text{mL}$ of Tf-Ga or 100 to 500 $\mu\text{g}/\text{mL}$ of apo-transferrin (apoTf) or transferrin-iron (Tf-Fe) (●—●); Tf-Ga 200 $\mu\text{g}/\text{mL}$ (△—△); Tf-Ga 400 $\mu\text{g}/\text{mL}$ (■—■).

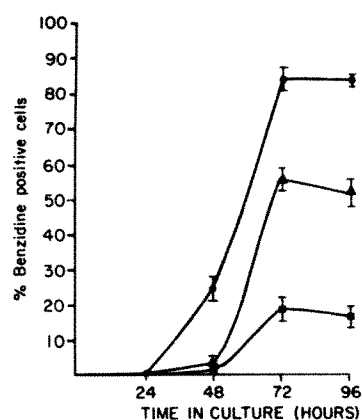


Fig 2. Effect of transferrin-gallium (Tf-Ga) on hemoglobin production by Friend erythroleukemia cells. Cells were plated in media containing 2% dimethylsulfoxide (DMSO) plus different transferrin forms and scored for benzidine positivity on days 1 through 4. No additives, 100 µg/mL of Tf-Ga or 100 to 500 µg/mL of apotransferrin (apoTf) or transferrin-iron (Tf-Fe) (●—●); Tf-Ga 200 µg/mL (▲—▲); Tf-Ga 400 µg/mL (■—■). Values represent the mean of three experiments $\pm$ SD.

further incubation led to a progressive decrease in cell viability, subsequent studies were done during the first 72 hours of cell growth.

To determine whether the decrease in hemoglobin production that occurred in cells exposed to Tf-Ga was the result of a decrease in proliferation per se, we incubated cells in 2% DMSO media containing concentrations of Ara-C that retarded cell growth and examined the effect on hemoglobin production. Ara-C has been reported to be a weak inducer of hemoglobin synthesis in some clones of Friend erythroleukemia cells; therefore, prior to using Ara-C to retard cell growth, we determined that this drug alone did not induce hemoglobin production (data not shown). As shown in Table 1, despite a decrease in cell growth to as low as 22% of

Table 1. Dissociation Between Hemoglobin Production and Inhibition of Cellular Proliferation

Media Additive (2% DMSO +)	Cell Count (% of Control)	(%) Benzidine- Positive Cells*
Control	100	85 $\pm$ 3.0
Ara-C		
.001 µg/mL	55	84 $\pm$ 3.0
.0075 µg/mL	45	77 $\pm$ 1.6
.01 µg/mL	22	76 $\pm$ 3.3
Tf-Ga		
100 µg/mL	100	85 $\pm$ 3.0
200 µg/mL	90	56 $\pm$ 3.2
400 µg/mL	68	19 $\pm$ 3.8
Ara-C .0075 µg/mL + Tf-Fe 100 µg/mL	45	76 $\pm$ 2.5
Ara-C .0075 µg/mL + Tf-Ga 100 µg/mL	45	39 $\pm$ 2.6

DMSO, dimethylsulfoxide; Ara-C, cytosine arabinoside; Tf-Ga, transferrin-gallium; Tf-Fe, transferrin-iron.

Cell counts and benzidine staining were performed in triplicate after 72 hours of incubation.

*Mean $\pm$ SD of three experiments.

control, cells exposed to Ara-C demonstrated benzidine positivity nearly equivalent to control. In contrast, decreases in cell growth to only 90% and 68% of control by Tf-Ga led to marked decreases in hemoglobin production. Furthermore, as shown in Table 1, when cell growth was controlled with Ara-C the addition of 100 µg/mL of Tf-Ga (a concentration that normally did not inhibit hemoglobin production) led to a marked decrease in hemoglobin production without a further decrease in cell growth. These studies suggest that the effects of Tf-Ga on cell growth and hemoglobin production are independent of each other and that the decrease in hemoglobin production following exposure of cells to higher concentrations of Tf-Ga is not directly due to decreases in the rate of cellular proliferation.

Maximum inhibition of hemoglobin production occurred when Tf-Ga was added at the time of initial plating, and the addition of Tf-Ga after cells had been growing in 2% DMSO media for 24 or 48 hours also significantly blocked further hemoglobin production. The addition of 400 µg/mL of Tf-Ga after 48 hours when >50% of the cells were benzidine positive actually resulted in a progressive decrease in the proportion of benzidine-positive cells over the subsequent 48 hours (Fig 3A). The removal of Tf-Ga from the media allowed cells to resume hemoglobin production, with the degree of recovery dependent on the time of Tf-Ga removal. Removal of Tf-Ga after the first 24 hours of incubation resulted in hemoglobin production equal to control cells, whereas removal of Tf-Ga after 48 or 72 hours led to lesser increases in hemoglobin-producing cells (Fig 3B). Cell growth and hemoglobin production beyond 96 hours could not be reliably assessed because of marked decreases in cell viability in all groups. These studies indicate that the inhibi-

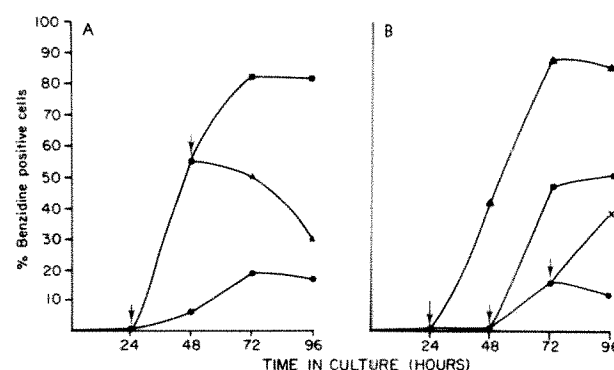


Fig 3. Hemoglobin production following addition of transferrin-gallium (Tf-Ga) to media (A) or removal of Tf-Ga from media (B) at different times. A: Friend erythroleukemia cells were plated in media containing 2% dimethylsulfoxide (DMSO); 400 µg/mL of Tf-Ga was added after 24 or 48 hours of incubation (arrows). Hemoglobin production is shown (benzidine positivity) with Tf-Ga added at 24 hours (●—●) and 48 hours (▲—▲) or with control (no Tf-Ga) (■—■). A representative experiment is shown. B: Friend erythroleukemia cells were plated in media containing 2% DMSO plus 400 µg/mL of Tf-Ga. Aliquots of cells were removed at 24, 48, or 72 hours (arrows), washed free of Tf-Ga, and resuspended in fresh 2% DMSO media without Tf-Ga. Benzidine positivity was determined on days 1 through 4 following removal of Tf-Ga at 24 hours (▲—▲), 48 hours (■—■), and 72 hours (—X), or with control (Tf-Ga 400 µg/mL) (●—●). A representative experiment is shown.

tion of hemoglobin production by Tf-Ga is reversible and is dependent on the continuous presence of Tf-Ga in the media.

Cellular uptake of ^{59}Fe . Because normal hemoglobin production requires the presence of an adequate supply of iron to the erythron, we examined whether Tf-Ga might be inhibiting hemoglobin production by interfering with cellular iron utilization. ^{59}Fe uptake was determined over a 24- and 72-hour incubation period, times at which hemoglobin production was zero and maximum in the control cells. ^{59}Fe uptake by Tf-Ga-treated cells was compared with ^{59}Fe uptake by cells exposed to corresponding concentrations of apoTf. Although apoTf was used as a control in these experiments, it was undoubtedly converted to Fe-Tf by iron present in the media; hence, ^{59}Fe uptake by the control cells actually represents ^{59}Fe uptake in the presence of Fe-Tf. As shown in Table 2, radioiron incorporation into the Tf-Ga-treated cells was decreased by 57%, 58%, and 65% at 24 hours and 41%, 45%, and 57% at 72 hours for Tf-Ga concentrations of 100, 200, and 400 $\mu\text{g/mL}$, respectively.

It thus appears that following exposure of cells to Tf-Ga a decrease in cellular ^{59}Fe incorporation occurs as an early event before hemoglobin production is detectable. In addition, a decrease in cellular iron incorporation is also seen with lower concentrations of Tf-Ga (100 $\mu\text{g/mL}$) that do not significantly decrease hemoglobin production. These results suggest that the progressive inhibition of hemoglobin production seen with higher doses of Tf-Ga results from decreased availability of iron to the cells.

Coincubation studies. Because Tf-Ga appeared to decrease the cellular uptake of trace amounts of ^{59}Fe , we examined whether the addition of exogenous Tf-Fe to the 2% DMSO-Tf-Ga media could reverse the inhibitory effects of Tf-Ga on cellular hemoglobin production. As shown in Figs 4A and B, the number of hemoglobin-producing cells increased to equal control values following the addition of Tf-Fe in concentrations equivalent to the Tf-Ga present in the media. We also noted that restoration of hemoglobin production occurred only with Tf-Fe and not with apoTf, indicating that the Tf-Ga effect resulted from specific interference with cellular iron utilization.

Recent studies using reticulocytes have demonstrated that Fe-PIH can provide iron for heme synthesis without using transferrin-transferrin receptor as a mediator.¹⁰ Therefore, in an attempt to exclude Tf-Fe and Tf-Ga interactions at the

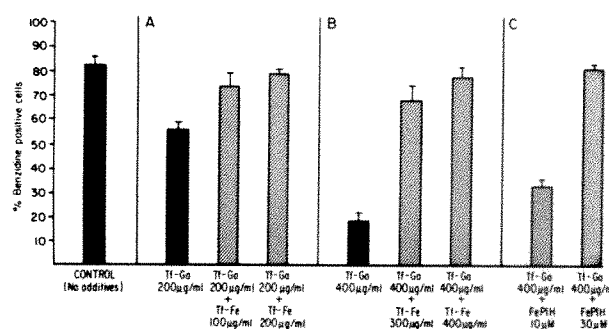


Fig 4. Effect of transferrin-iron (Tf-Fe) or iron-pyridoxal isonicotinoyl hydrazone (Fe-PIH) on inhibition of hemoglobin production by transferrin-gallium (Tf-Ga). Cells were plated in media containing 2% dimethylsulfoxide (DMSO) plus the different additives, and benzidine positivity was determined after 72 hours of culture. (A) Tf-Fe 100 or 200 $\mu\text{g/mL}$ was added to media containing 200 $\mu\text{g/mL}$ of Tf-Ga. (B) Tf-Fe 300 or 400 $\mu\text{g/mL}$ was added to media containing 400 $\mu\text{g/mL}$ of Tf-Ga. (C) Fe-PIH 10 or 30 $\mu\text{mol/L}$ was added to media containing 400 $\mu\text{g/mL}$ of Tf-Ga. Bars represent the mean of 3 to 6 experiments $\pm$ SD.

cell surface transferrin receptor level, we examined whether iron provided by Fe-PIH could overcome the inhibitory effect of Tf-Ga on cellular hemoglobin production. As shown in Fig 4C, the addition of 30 $\mu\text{mol/L}$ of Fe-PIH resulted in the return of hemoglobin-producing cells to control levels. The amount of Fe required to return hemoglobin production to normal was higher when Fe was provided as Fe-PIH (30 $\mu\text{mol/L}$) than when Fe was provided as Tf-Fe (400 $\mu\text{g/mL}$ of Tf-Fe = 10 $\mu\text{mol/L}$ Fe). This most likely reflects differences in the ability of the two Fe forms (Tf-Fe or Fe-PIH) to provide iron to the cells.

Transferrin receptor expression. ^{125}I -Transferrin binding studies were performed on cells after growth for different lengths of time in regular media, 2% DMSO media alone, or with Tf-Ga in concentrations of 200 and 400 $\mu\text{g/mL}$. Figure 5 shows that uninduced cells in regular media reached a plateau in transferrin receptor expression between 24 and 48 hours, followed by a progressive decrease in transferrin receptor expression. Induced cells (2% DMSO) displayed a progressive increase in transferrin receptor expression corresponding to their increase in hemoglobin production. In contrast, exposure of cells to 2% DMSO plus Tf-Ga led to even greater increases in transferrin receptor expression after 24, 48, and 72 hours of incubation, despite significant decreases in hemoglobin production.

DISCUSSION

We demonstrated that Tf-Ga is able to inhibit hemoglobin production in Friend erythroleukemia cells by specifically interfering with the cellular incorporation of iron. Our *in vitro* studies suggest that such a mechanism is involved *in vivo* during gallium nitrate therapy and that decreased iron incorporation into maturing erythroid precursors leads to the development of a microcytic hypochromic anemia characteristic of iron-deficient erythropoiesis. In preliminary studies, we noted that hemoglobin production by Friend erythroleukemia cells could be inhibited by the addition of gallium nitrate to tissue culture media. Under these conditions,

Table 2. Cellular Uptake of ^{59}Fe in the Presence of apoTf or Tf-Ga

Media Additive* ($\mu\text{g/mL}$)	^{59}Fe Uptake pg/ 10^6 Cells†	
	24 hours	72 hours
ApoTf (100)	399 $\pm$ 2.5	277 $\pm$ 12.2
Tf-Ga (100)	172 $\pm$ 5.6	164 $\pm$ 8.6
ApoTf (200)	325 $\pm$ 11.6	227 $\pm$ 29.2
Tf-Ga (200)	137 $\pm$ 9.9	126 $\pm$ 13.4
ApoTf (400)	306 $\pm$ 31.7	207 $\pm$ 15.0
Tf-Ga (400)	107 $\pm$ 4.7	90 $\pm$ 10.3

ApoTf, apotransferrin; other abbreviations as in Table 1.

*Media also contained 2% DMSO.

†Data represent the mean $\pm$ SD of 3 to 6 experiments.

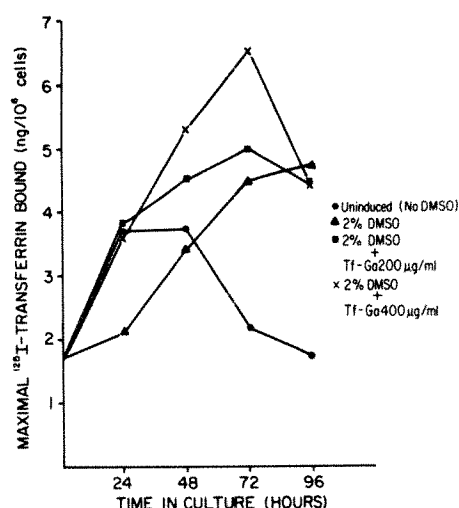


Fig 5. Measurement of cellular ^{125}I -Tf binding sites following cell growth in regular media, 2% dimethylsulfoxide (DMSO) media or 2% DMSO media plus transferrin-gallium (Tf-Ga). Friend erythroleukemia cells were plated at $1 \times 10^6/\text{mL}$ in the different media; after 24, 48, 72, or 96 hours, cells were harvested, receptor-bound transferrin was eluted, and assays for Tf binding were performed. Maximal Tf binding was determined. All points represent the mean of duplicate determinations in two separate experiments.

gallium rapidly binds to bovine transferrin present in the media to form Tf-Ga. A similar sequence of events most likely occurs *in vivo*: ie, gallium binds to transferrin, and the Tf-Ga complex is then able to inhibit iron uptake by erythroid precursors significantly. *In vivo*, normally only one-third of plasma transferrin is iron saturated; therefore, the remainder is free to bind gallium avidly. With the formation of increasing concentrations of Tf-Ga, iron uptake by the erythron is impaired, and significant anemia can develop.

To control the experimental conditions and the concentration of Tf-Ga in the media better, we prepared Tf-Ga and examined its effect on hemoglobin production, cellular incorporation of ^{59}Fe , and expression of transferrin receptors. We noted that Tf-Ga markedly inhibited hemoglobin production. This inhibition of hemoglobin production was independent of the mild-to-moderate decrease in cell growth seen following exposure of cells to Tf-Ga, since a decrease in hemoglobin production was not seen when cellular proliferation was markedly inhibited by Ara-C. In addition, when cell growth was controlled with Ara-C and the effects of Tf-Ga and Tf-Fe were compared, low concentrations of Tf-Ga inhibited hemoglobin synthesis without decreasing cellular proliferation. Furthermore, removal of Tf-Ga from the media led to a rapid increase in cellular hemoglobin production, indicating that a reversible noncytotoxic mechanism was involved.

Our ^{59}Fe uptake, coincubation, and transferrin receptor expression studies demonstrate that Tf-Ga inhibits cellular hemoglobin production by interfering with cellular iron incorporation. Decreases in cellular ^{59}Fe uptake were seen with concentrations of Tf-Ga that inhibited hemoglobin production and also with concentrations of Tf-Ga that did not affect hemoglobin production. These results suggest that

a decrease in cellular iron uptake is one of the first events to occur after exposure to Tf-Ga. Higher concentrations of Tf-Ga further inhibit cellular iron uptake, resulting in decreased hemoglobin production. The coincubation studies confirm that a decrease in cellular iron incorporation is responsible for the decreased hemoglobin production, since the addition of Tf-Fe or Fe-PIH to the media reversed Tf-Ga inhibition of hemoglobin production. Recent studies have demonstrated that Fe-PIH can deliver iron to reticulocytes for heme synthesis even after cell surface transferrin receptors have been removed by pronase treatment.¹⁰ Hence, the restoration of hemoglobin production by Tf-Fe or Fe-PIH in cells exposed to Tf-Ga appears to be the result of delivery of iron to the cell interior, and not to the cell surface alone.

The marked up-regulation of transferrin receptor density seen on Tf-Ga-treated cells is compatible with decreased iron incorporation into these cells. Whereas the progressive increase in transferrin receptor expression on induced cells corresponds with increased iron requirements for hemoglobin synthesis, transferrin receptor up-regulation on cells exposed to Tf-Ga reflects an attempt by these cells to overcome the block in iron incorporation. Indeed, cells that incorporated the least amount of ^{59}Fe and thus produced the least amount of hemoglobin following exposure to Tf-Ga were the ones that displayed the highest density of transferrin receptors. This increase in transferrin receptor expression in response to decreased iron availability is consistent with other studies of transferrin receptor regulation in which an up-regulation of the transferrin receptor is seen following exposure of cells to various iron chelators that limit iron availability.¹⁹⁻²¹

The mechanism whereby Tf-Ga inhibits cellular iron incorporation is currently being investigated. Our studies with human promyelocytic leukemic HL60 cells suggest that Tf-Ga-treated cells tend to release iron into the media after the initial binding and internalization steps,²² and that Tf-Ga inhibition of cellular iron uptake is not due merely to competitive binding of Tf-Ga to cell surface transferrin receptors.

Gallium appears to be a unique chemotherapeutic agent that binds to transferrin; this may result in "targeting" to certain tumors such as lymphomas, which express transferrin receptors *in vivo*²³ and are responsive to treatment with gallium nitrate.² Blockade of the transferrin receptor by an anti-transferrin receptor monoclonal antibody has been demonstrated to inhibit tumor cell growth in a nude mouse model.²⁴ Similar targeting of the Tf-Ga complex to erythroid precursors would lead to inhibition of iron incorporation into these cells and the development of a microcytic hypochromic anemia. Because the cytotoxicity of gallium appears to be due, at least in part, to inhibition of cellular iron incorporation, this interaction between iron and gallium should be kept in mind during current trials with gallium nitrate. It remains to be determined whether gallium can influence other intracellular iron-dependent processes. Tf-Ga may provide an interesting tool for examining the shunting of iron from heme, ferritin, or other intracellular compartments. Studies of the intracellular interaction between iron and gallium might "unmask" steps at which iron is required for cellular proliferation.

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Characterization of the Inhibition of Tissue Factor in Serum

By George J. Broze, Jr and Joseph P. Miletich

Tissue factor (TF) is a lipoprotein cofactor that markedly enhances the proteolytic activation of factors IX and X by factor VIIa. The functional activity of TF is inhibited by serum in a time- and temperature-dependent fashion. The inhibitory effect is also dependent on the presence of calcium ions and can be reversed by calcium chelation (EDTA) and dilution, thus excluding direct proteolytic destruction of TF as the mechanism for inhibition. Using crude TF, serum immunodepleted of factor VII, and serum depleted of the vitamin K-dependent coagulation factors by BaSO₄ absorption, it is shown that TF factor inhibition requires the presence of VII(a), X(a), and an additional moiety contained in barium-absorbed serum. When each of the other required components were at saturating concentrations, half-maximal inhibition of TF occurred in reaction

mixtures containing 2% (vol/vol) of TF at a factor VII(a) concentration of 4 ng/mL (80 pmol/L), a factor X concentration of 50 ng/mL (850 pmol/L), and a concentration of barium-absorbed serum of 2.5% (vol/vol). Catalytically active factor Xa appeared to be required for the generation of optimal TF inhibition. The results are consistent with the conclusions of Hjort that barium-absorbed serum contains a moiety that inhibits the VIIa-Ca²⁺-TF complex. The role of factor X(a) in the generation of the inhibitory phenomenon remains to be elucidated. The inhibitor present in serum (plasma) may in part be produced by the liver in vivo since cultured human hepatoma cells (HepG2) secrete this inhibitory activity in vitro.

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TISSUE FACTOR (TF) is a lipoprotein cofactor that triggers blood coagulation by markedly enhancing the catalytic activity of factor VIIa toward its substrates, factor X and factor IX.^{1,2} When plasma is induced to clot by the addition of small amounts of TF, the presence of factors VIII and IX appear to be required for optimum coagulation.^{3,4} The studies of Marlar and colleagues have shown that, under these circumstances (small quantities of TF), only a limited amount of activation of factor X to Xa occurs in the absence of factor VIII or IX.⁵ Although not mentioned by these authors, this implies the generation of an inhibitor in plasma that affects the activities of VII(a), TF, or the VII(a)-TF complex. Later, the same phenomena was demonstrated by Morrison and Jesty and shown to be related to the inclusion of factor X(a) in the reaction mixtures.⁶ Very recently, Sanders and co-workers confirmed the requirement for factor X(a) and found that an additional moiety in barium-absorbed plasma was also required for the generation of this inhibitory effect.⁷

These recent articles have rediscovered a phenomenon that had previously been studied extensively by several investigators.⁸⁻²² Using a specific two-stage TF assay, we show that the inhibitor most likely recognizes the TF-Ca²⁺-VII(a) complex, appears to require the presence of

catalytically active X(a), and is reversible, thus excluding a direct proteolytic effect on tissue factor.

MATERIALS AND METHODS

Materials

Affigel 10 was purchased from Bio Rad Laboratories (Richmond, CA) and bovine serum albumin (BSA), Trizma base, acrylamide, bisacrylamide, kaolin, rabbit brain cephalin, and RVV/cephalin, were bought from Sigma Chemical (St Louis). Sodium ¹²⁵I-iodide, carrier-free, was purchased from New England Nuclear (Boston) and Iodo-gen was obtained from Pierce Chemical (Rockford, IL). Diisofluorophosphate (DFP) was a product of Aldrich Chemical (Milwaukee). All other chemicals were reagent grade or better and came from Sigma or from Fisher Scientific (Pittsburgh). HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD).

Serum. Blood-serum was produced by incubating blood drawn in the absence of anticoagulants in glass tubes for 2 hours at 37°C. The clot was then gently rimmed, and supernatant serum was obtained following centrifugation (4,000 g for 10 minutes). Plasma-serum was produced from pooled normal plasma (George King Biomedical, Overland Park, KS) by adding to the citrated plasma 1/10 vol of kaolin 4 g/dL in normal saline, 1/100 vol of stock rabbit brain cephalin (prepared according to the manufacturer), and 1/100 vol 2.5 mol/L of CaCl₂. After mixing, the plasma was allowed to clot for 2 hours at 37°C and was then centrifuged (4,000 g for 10 minutes). The supernatant serums were decanted, dialyzed at 4°C against 100 vol of 0.1 mol/L of NaCl, 0.05 mol/L of Tris-HCl, and 0.1% NaN₃, pH 7.5 (TS) with two changes, and stored at 4°C. Due to the dilutions associated with its preparation, we estimate that the final plasma-serum reagent had a serum concentration that was 74% that of blood-serum.

For BaSO₄ absorption, serum was dialyzed against TS with 0.01 mol/L of sodium oxalate. BaSO₄ (100 mg/mL) was then added to the serum; after mixing for 30 minutes at room temperature, the BaSO₄ was removed by centrifugation and the absorbed serum was once again dialyzed into TS buffer. This BaSO₄-absorbed serum contained <1% factor X and 1% factor VII activity of the starting serum.

Factor VII-depleted serum was produced by passing normal human serum through a column of mouse monoclonal anti-factor VII IgG linked to affigel 10 (MC 1476) as previously described.²³ Under these conditions, the factor VII-depleted serum contained ~0.9% of the factor VII activity of the starting serum.

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Proteins

X coagulant protein (XCP) from Russell's viper venom (RVV), factor VII, and factor X were purified as previously described.²⁴⁻²⁶ Factor VIIa was purified from Cohn III paste. Extraction of the Cohn III paste, polyethylene glycol precipitation, and barium citrate precipitation were performed as described by Fenton and co-workers.²⁷ The factor VII(a) was then isolated from the barium citrate pellet by ammonium sulfate elution and fractionation, monoclonal immunoaffinity chromatography, and ACA 44 gel filtration as previously described.²⁵ The final preparation contained 35% single-chain factor VII and 65% two-chain factor VIIa as determined by densitometry of the Coomassie brilliant blue R-stained protein following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. It had a specific factor VII activity of 50 U/ μ g. For the purposes of the experiments reported here, this preparation is called VIIa.

Factor Xa was produced by incubation of purified factor X with insolubilized XCP as previously described.²⁸ DFP-treated (DIP)-factor Xa was made by incubating factor Xa with 5 mmol/L of DFP for 48 hours at 4 °C. Under these conditions, DIP-factor Xa possessed <1% the specific activity of the starting factor Xa, but still possessed 3% (wt/wt) factor X when "total" factor X activity (described below) was determined. This implied that the activation of factor X by the insolubilized XCP was incomplete (97%) and, indeed, SDS-PAGE of 25 μ g of this Xa preparation showed that it contained a trace amount of unactivated factor X.

Crude TF was prepared as previously described, and EDTA-TF was produced by extensive washing of the crude preparation with TS containing 5 mmol/L of EDTA using ultracentrifugation (105,000 g for 1 hour) and final resuspension of the pellet containing the TF activity in TS.²⁹ In a single-stage assay using normal human plasma (100 μ L), diluted EDTA-TF (100 μ L), and CaCl_2 25 mmol/L (100 μ L) a dilution of 1:1600 of this crude TF preparation gave a clotting time of 32 seconds.

Functional assays. Factor VII and factor Xa assays were performed as previously described.²⁹ Total factor X activity was determined by incubating RVV/cephalin reagent (60 μ L), the sample in TS with 1 mg/mL of BSA (TBSA) (60 μ L), and CaCl_2 25 mmol/L (60 μ L) at 37 °C. After 3 minutes, factor X-deficient plasma was added, and the time to clot formation was determined using a fibrometer (BBL, Cockeysville, MD).

A three-stage assay was developed to assay for TF inhibition (TFI). In the first stage, serum (50% vol/vol), CaCl_2 (4 mmol/L), TBSA, or other additions (eg, VIIa, X, or Xa), and EDTA-TF were incubated at room temperature unless otherwise noted. At specified times, 10- μ L samples were diluted in TBSA with 5 mmol/L of CaCl_2 or TBSA with 1 mmol/L of EDTA and assayed for TF activity using a previously described two-stage assay system.²⁵ Fifty microliters of the diluted sample (final dilution in assay 500- to 50,000-fold), 50 μ L of factor VIIa (1 μ g/mL in TBSA), 50 μ L of 25 mmol/L of CaCl_2 , and 50 μ L of factor X (10 μ g/mL in TBSA) were incubated at 37 °C. After 1 minute, 50 μ L of a mixture containing ten parts of factor X-deficient plasma and one part of rabbit brain cephalin stock reagent (prepared as described by Sigma) was added, and the time to clot formation was determined with a fibrometer (BBL). For the purposes of comparing the effect of various additions in the first stage of the assay, 100% TF activity was defined as the activity present when only TF and TBSA comprised the first stage.

SDS-PAGE was performed on 10% gels (ratio acrylamide:bis; 37.5:1) by the method of Laemmli (4% stacking gel).³⁰ Samples were boiled in the presence of 10% 2-mercaptoethanol prior to electrophoresis.

Cell culture. HepG2 cells were cultured as previously described.²³ To assess the secretion of inhibitory activity, adherent

cells were washed twice with serum-free media and then incubated in serum-free media for 3 days.

RESULTS

When crude, EDTA-washed, TF (2% vol/vol) was incubated with serum derived from blood (50% vol/vol) in the presence of CaCl_2 there was a very rapid reduction in apparent TF activity as measured in the two-stage assay (described in the Materials and Methods section) following dilution of subsamples into a buffer containing 5 mmol/L CaCl_2 . Essentially no TF inhibition could be demonstrated, however, when subsamples of the same mixtures were diluted in a buffer containing EDTA (1 mmol/L) prior to functional assay, or if EDTA (5 mmol/L) was present in the reaction mixture from the onset of the incubation (Fig 1). The inhibition occurred at a much slower rate when the reaction mixture was incubated at 4 °C v 20 °C or 37 °C (not shown).

These results suggested that the inhibitory moiety present in serum might recognize the factor VII(a)-TF complex, which requires the presence of Ca^{2+} , and disassociates following chelation with EDTA.³¹ The reversibility of TFI with calcium chelation also meant that the inhibition was not due to enzymatic destruction of TF itself.

Effect of VII(a) Concentrations on TF Inhibition

To confirm that factor VII(a) was required for the generation of this apparent TFI, an additional experiment was performed. Plasma-serum (50% vol/vol), depleted of factor VII(a) by affinity chromatography (residual VII activity 0.9%), was tested for its ability to inhibit TF (2% vol/vol) in

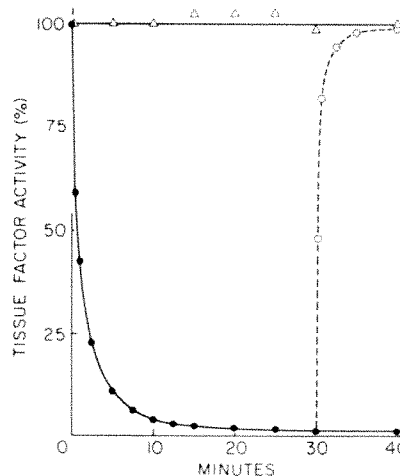


Fig 1. Inhibition of tissue factor (TF) in serum. A mixture containing 50% (vol/vol) blood serum, 2% (vol/vol) TF and 4 mmol/L of CaCl_2 was incubated at room temperature. Samples were removed at indicated times, diluted into buffer containing 5 mmol/L of CaCl_2 and assayed for TF activity (●—●). At 30 minutes, a sample was also diluted in buffer containing 1 mmol/L of EDTA, and the apparent TF activity determined at specific times following dilution (○—○). The TF activity remaining in an identical reaction mixture containing 5 mmol/L of EDTA rather than 4 mmol/L of CaCl_2 from the onset was also determined (dilutions into buffer containing CaCl_2) (Δ—Δ).

the presence of varying amounts of exogenous VIIa. Figure 2 shows that with increasing concentrations of VIIa, increasing TFI occurred. Furthermore, 50% inhibition of the TF occurred at concentrations of added VIIa between 1.25 and 2.5 ng/mL. Taking into account the residual VII(a) in the depleted serum (~4 ng/mL), which was 50% vol/vol of the sample, this corresponds to a total concentration of VII(a) in the incubation mixtures of 3.25 to 4.45 ng/mL. These VII(a) concentrations are remarkably similar to those required to half-saturate TF-binding sites on monocytes in a purified system.³¹ Direct comparison between the kinetic data derived from the purified system and the results in the complex mixtures containing serum and a crude brain homogenate as a source of TF used must, however, be made with caution. It is clear from Fig 2 that the TFI appears to reach a plateau by ~30 minutes of incubation.

Effect of X Concentrations on TFI

To assess the requirement of factor X for TFI noted previously by Morrison and Jesty,⁶ and Sanders and co-workers,⁷ plasma-serum was BaSO₄ absorbed. Reaction mixtures containing BaSO₄-absorbed plasma-serum (50% vol/vol), added VIIa (final concentration 100 ng/mL), and varying concentrations of X were incubated for 30 minutes, and the degree of TFI was determined. Figure 3 shows the relationship between TFI and concentrations of exogenous, added X in the reaction mixtures. By extrapolating the curve to 0 TFI, we estimate that the barium-absorbed serum contributed an additional 12.5 ng/mL of X to the reaction mixtures, which agrees well with the results of the functional assays of the serum before and after BaSO₄ absorption. The insert to Fig 3 shows the double-reciprocal plot of 1/TFI v 1/total X (X remaining in the barium-absorbed serum plus exogenously added X). Fifty percent inhibition occurred at a total X concentration of 50 ng/mL in the reaction mixture.

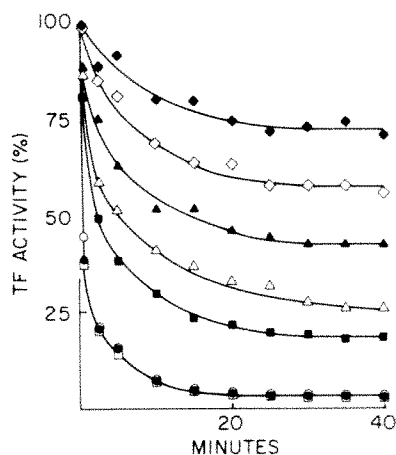


Fig 2. Effect of factor VIIa on tissue factor (TF) inhibition. Mixtures containing 50% (vol/vol) plasma-serum immunodepleted of factor VII(a), 2% (vol/vol) TF, 4 mmol/L of CaCl₂, and various concentrations of exogenously added factor VIIa were incubated at room temperature. Remaining TF activity was determined: no added factor VIIa (●—●); 1.25 ng/mL (◇—◇); 2.5 ng/mL (▲—▲); 5 ng/mL (△—△); 10 ng/mL (■—■); 50 ng/mL (○—○); and 100 ng/mL (●—●) of factor VIIa. Plasma-serum prior to immunodepletion of factor VII (a) (□—□).

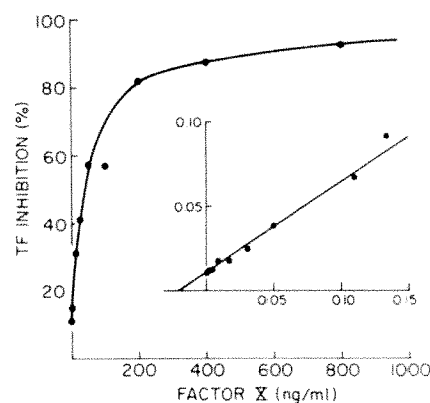


Fig 3. Effect of factor X on tissue factor inhibition (TFI). Mixtures containing 50% (vol/vol) BaSO₄-absorbed plasma-serum, 2% (vol/vol) TF, 100 ng/mL of factor VIIa, and various concentrations of exogenously added factor X were incubated at room temperature for 30 minutes, and the degree of TFI was determined. Inset: y axis, 1/TFI; x axis, 1/total factor X concentration in the reaction mixture. Total factor X is the sum of the amount of exogenous factor X added plus the estimated factor X remaining in the BaSO₄-absorbed serum.

Effect of Serum Concentrations on TFI

To confirm that TFI in serum required not only VII(a) and X(a) but an additional moiety present in barium-absorbed serum, the experiment depicted in Fig 4 was performed. The degree of TFI in mixtures containing VIIa and X (100 ng/mL and 1 μg/mL, respectively), 2% (vol/vol) TF, and varying concentrations of BaSO₄-absorbed plasma-serum was determined. Under these conditions, 50% TFI occurred at a concentration of barium-absorbed serum of 2.5% (vol/vol).

Requirement for Catalytically Active Xa

Reaction mixtures containing 2% (vol/vol) TF and 50% (vol/vol) plasma-serum were prepared containing X, Xa, or

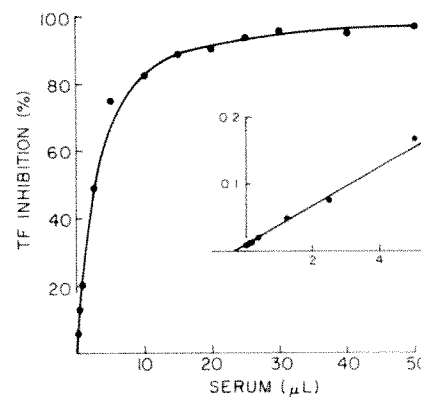


Fig 4. Effect of serum concentration on tissue factor inhibition (TFI). Mixtures (total volume 100 μL) containing 2% (vol/vol) tissue factor, 100 ng/mL of factor VIIa, 1 μg/mL of factor X, 4 mmol/L of CaCl₂, and varying amounts of BaSO₄-absorbed plasma-serum were incubated for 30 minutes at room temperature and the degree of TFI was determined. Inset: y axis, 1/TFI; x axis, 1/serum (μL).

DIP-Xa (250 ng/mL) in the presence or absence of VIIa (100 ng/mL). The extent of TFI was determined following 30 minutes of incubation and is presented in Table 1. It is evident that the addition of both VIIa and X or Xa was required to generate optimum TFI, whereas Xa alone was insufficient (+VIIa v -VIIa columns, Table 1). Furthermore, in mixtures containing additional VIIa, DIP-factor Xa was less effective than X(a) (22.1% v 89.1% TFI) with the degree of TFI detected in the sample containing DIP-factor Xa probably related to contaminating factor X (3% wt/wt) in the preparation (19.2% TFI).

Production of TF Inhibitory Activity by Human Hepatoma Cells

It has been suggested that the inhibitor of the VII-TF complex may be a lipoprotein,^{7,32} and we noted that the serum inhibitory activity is found in the total lipoprotein fraction following centrifugation of serum at a density of 1.21 g/ml (data not shown).³³ In view of this, it seemed likely that the liver might be a potential source of this inhibitory agent. Therefore, the serum-free conditioned media from human hepatoma cells (HepG2) cultured in vitro was tested for TF inhibitory activity in the three-stage assay described in the Materials and Methods section. The results (Fig 5) indeed showed substantial apparent inhibitory activity in the conditioned media that increased progressively over the time period examined.

DISCUSSION

The apparent inhibition of tissue thromboplastin (TF) by serum was studied extensively by early investigators.⁸⁻²² Perhaps this effect was most dramatically demonstrated by experiments showing that prior incubation of tissue thromboplastin with serum prevented its mortal effect when infused into mice.¹⁰⁻¹⁵ Additional experiments demonstrated that the inhibition of thromboplastin was calcium dependent, reversible, and exhaustible.¹⁵⁻¹⁹ These results were confirmed and extended in elegant studies performed by Hjort.²⁰ He concluded that barium-absorbed serum contained an inhibitor that recognized "convertin" [TF-Ca²⁺-VII(a)], but had no effect on "proconvertin" [VII(a)] or thromboplastin (TF). Because Hjort's proconvertin reagent also contained factor X(a), he was unable to discern the more recently described requirement of X(A) for the inhibition.^{6,7}

Table 1. Effect of X, Xa, and DIP-Xa on Tissue Factor Inhibition in BaSO₄-Absorbed Plasma-Serum

X Preparation Added	Tissue Factor Inhibition (%)*	
	+ VIIa†	- VIIa
X (250 ng/mL)‡	89.0	19.6
Xa (250 ng/mL)	89.1	22.5
DIP-Xa (250 ng/mL)	22.1	19.6
X (7.5 ng/mL)	19.2	19.6
None	13.5	13.4

DIP = Xa, diisofluorophosphate-treated factor Xa.

*Results are the mean of duplicate experiments.

†Exogenous VII added to a final concentration of 100 ng/mL in incubation mixture.

‡Final concentration of X preparation in incubation mixture.

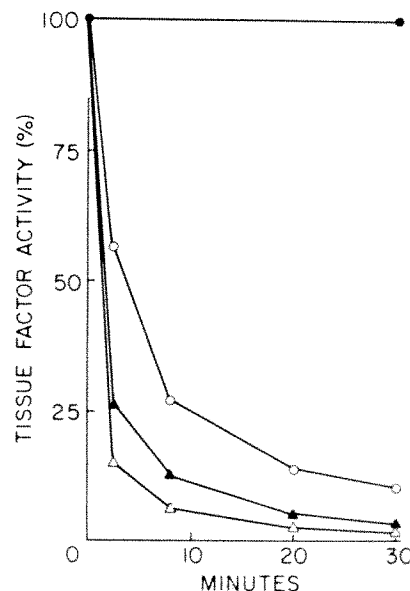


Fig 5. Secretion of inhibitory activity by HepG2 cells in vitro. HepG2 cells were grown to confluence in media containing 10% fetal calf serum. The serum-containing media was then removed, and the cells were washed twice with serum-free media before subsequent incubation in serum-free media. Samples of the serum-free-conditioned media were removed on day 1 (○—○), 2 (▲—▲), and 3 (△—△), and tested for the ability to inhibit tissue factor (TF) in mixtures containing 50% (vol/vol) conditioned media, 2% (vol/vol) TF, 100 ng/mL of factor VIIa, 1 µg/mL of factor X, and 4 mmol/L of CaCl₂ incubated at room temperature. Serum-free media control (●—●).

Investigating the activation of ³H-labeled X or IX in specifically depleted plasmas on the addition of TF, Morrison and Jesty confirmed previous work by Marlar and co-workers showing that the extent of activation of X (and IX) was incomplete.^{5,6} The inclusion of X or Xa in their reaction mixtures appeared to be associated with the ultimate plateau in X and IX activation. They hypothesized that Xa might proteolytically destroy the VII(a) present since, in their hands, the addition of further TF did not cause further activation of X. Sanders and colleagues, who also studied the activation of X and IX in plasma using the release of ³H activation peptides, found that the addition of further TF to a reaction mixture in which factor X activation had plateaued produced further factor X activation, implying that the apparent inhibition of factor X activation was not due to proteolytic destruction of all the VII(a) in plasma.⁷ They suggested instead, that the inhibitory effect was related to the destruction of TF.

Our results, however, in agreement with those of Hjort, suggest that the inhibitory factor present in barium-absorbed serum reacts with the factor VII(a)-Ca²⁺-TF complex and does not destroy TF per se since TF functional activity can be recovered following chelation of Ca²⁺ and dilution.³¹ Rao and colleagues, using different methods, recently reached a similar conclusion.³⁴ Furthermore, our studies make it unlikely that the inhibition of factor X activation by VIIa-TF is due to proteolytic destruction of VIIa since it persisted following dilution and the addition of more VIIa (steps used to

measure TF activity here). In another experiment (not shown), ^{125}I -radiolabeled VII (4 ng/mL) was incubated with 50% (vol/vol) immunodepleted factor VII-deficient plasma-serum, and 20% (vol/vol) TF. Samples were removed following incubation of the mixture at room temperature, subjected to SDS-PAGE under reducing conditions, and autoradiographed. Under these conditions, cleavage of ^{125}I -VII to two-chain factor VIIa was observed, but no additional proteolysis of VII could be detected.

To produce optimal TF inhibition in BaSO_4 -adsorbed serum, both VIIa and X(a) were required. Thus, the role of VIIa in the mixtures was not simply the activation of X since Xa alone was insufficient. Furthermore, the function of Xa was not simply the activation of VII since the addition of VIIa (our preparation contained 65% two-chain, activated VII) to BaSO_4 -adsorbed serum was also insufficient to produce maximal TF inhibition. Although the indirect evidence presented here strongly supports the supposition that the inhibitor recognizes the VII(a)- Ca^{2+} -TF complex, thus explaining the requirement for VII(a), the role of X(a) remains to be elucidated. The results depicted in Table 1 suggest that catalytically active Xa is required for some step in the generation of the inhibitory activity in serum and, in additional studies (not shown), we found that complexes of Xa-antithrombin III (or Xa-soybean trypsin inhibitor), will not suffice. With this in mind, and because serum contains inhibitors to Xa, it appears likely that the concentration of X(a) required for 50% TF inhibition as shown in Fig 3 (50 ng/mL) is probably an overestimate. Whether a stoichiomet-

ric complex of VII(a)-TF-Xa might be involved in the inhibitory process, however, will require isolation of the factor present in barium-adsorbed serum (plasma) to allow experiments in purified systems.

Carson has reported that high-density lipoproteins (HDLs) inhibit the activation of factor X by factor VIIa and TF.³² We found, however, that HDL isolated using polyanion precipitation and density centrifugation³⁵ did not affect TF activity when measured in the assay system described here. Thus, the inhibition noted by Carson may involve a separate mechanism from that of the inhibitor of VIIa-TF we are studying. Kondo and Kisiel have recently described the isolation of a lipoprotein that inhibits factor VIIa-TF activation of factor X.³⁶ This moiety copurified with factor X through a number of chromatography steps and was found to inhibit the activation of X by the VIIa-TF complex in a noncompetitive fashion, suggesting to them that its mechanism of action involves the sequestration of either VII(a) or X. The relationship between this lipoprotein and the serum factor associated with apparent TFI that is the subject of this report is unclear. Moreover, although the characteristics of the latter inhibitor are compatible with noncompetitive inhibition, our results appear to make the sequestration of VIIa or X per se an unlikely mechanism of its action.

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Association of Red Cell Spherocytosis With Deletion of the Short Arm of Chromosome 8

By Robert R. Chilcote, Michelle M. Le Beau, Carlton Dampier, Eugene Pergament, Yury Verlinsky, Narla Mohandas, Henri Frischer, and Janet D. Rowley

Congenital spherocytic anemia is a common disorder, but in most cases the nature of the underlying membrane lesion is unknown and the genetic defect has not yet been unequivocally mapped to a chromosome. We studied two dysmorphic siblings with neurologic findings and hemolytic anemia. Clinical and laboratory findings in these two siblings were consistent with the diagnosis of congenital spherocytosis whereas both parents and two unaffected

siblings were normal. The two affected children had an abnormal chromosomal complement as a result of a deletion of the short arm of chromosome 8 [(46,XX,del(8)(p11.1p21.1)]. These results suggest that a gene whose deletion results in a congenital spherocytic anemia phenotype resides on this region on the short arm of chromosome 8.

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CONGENITAL or heritable spherocytosis (HS) is a hemolytic anemia of variable severity, ordinarily inherited in an autosomal dominant fashion and alleviated by splenectomy. Although HS is associated with RBC membrane loss that results in characteristic findings in osmotic fragility studies, in the majority of cases studied, no consistent molecular membrane defect has been identified and no study has unambiguously assigned the defective gene(s) to a chromosome. Kimberling and associates^{1,2} described a family with both HS and a balanced translocation between chromosomes 8 and 12, whereas Bass and associates³ described another family with HS and a translocation between chromosomes 3 and 8, suggesting that a functional alteration in a gene on chromosome 8 would result in spherocytosis. We report two sisters with typical spherocytosis, dysmorphology, and an interstitial deletion of the short arm (p) of chromosome 8.

MATERIALS AND METHODS

Case reports. The family was first brought to medical attention because of anemia in C.H. (Table 1). Born in 1978, this child was small (birth weight 2,500 g; length 38 cm), had hyperbilirubinemia

in the second day of life and gained weight slowly. In the first months of life, intermittent splenomegaly, anemia, reticulocytosis, and microspherocytes in the peripheral smear were noticed. When the child was aged 6 months, psychomotor retardation, unusual facies, and nystagmus became evident and tonic-clonic seizures occurred. The results of osmotic fragility studies of RBCs before and after 24-hour incubation were characteristic of spherocytosis. The patient required several transfusions following respiratory infections in the first years of life. After splenectomy was performed when she was aged 4 years, her hemoglobin levels and reticulocyte counts have been normal and transfusions have not been necessary. However, microspherocytes persist on the peripheral smear, and osmotic fragility studies of both fresh and incubated erythrocytes continue to show increased fragility.

The second child, born in 1980, was also small at birth (birth weight 2,700 g; length 41 cm); she developed similar dysmorphic features with nystagmus, psychomotor retardation, and intermittent splenomegaly. Hematologic studies revealed that she also has spherocytosis. Anemia was not as severe as in the first child, and no transfusions have been required.

Both children attend schools for the educationally handicapped, have grown more slowly than their peers, and have a slight mongoloid slant, a pit located anterior to one ear, and micrognathia. The parents are unrelated and of normal appearance (mother's height 65 inches; father's height 79 inches) and are college graduates. There was no family history of congenital anomalies, anemia, jaundice, gall bladder disease, or splenectomy. On two occasions, the complete blood counts, blood films, serum bilirubins, haptoglobin levels, and osmotic fragility studies (before and after 24-hour incubation at 37 °C) on both parents were normal.

A third female child born in 1983 was normal (birth weight 3,540 g; length 45 cm) and by 3 years of age had not developed dysmorphic features, neurologic problems, or evidence of spherocytic anemia. A fourth child was born in 1986 and at 9 months of age shows normal development, is not dysmorphic, and has normal blood counts.

Laboratory studies. Using previously described techniques, RBC ektacytometric studies⁴ were performed, and carbonic anhydrase⁵ and glutathione reductase (GSSG-R) levels⁶ were measured on the two affected children and both parents. Plasma factor VII levels were measured using factor-deficient plasma (Dade Diagnostics). Peripheral blood RBCs were separated by density.⁷ Membrane proteins from RBC ghosts prepared by hypotonic lysis were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described⁸ (except 0.1% SDS gel and buffers were used to enhance resolution of band 4.1) and the two-dimensional gel technique of O'Farrell.⁹ One-dimensional gels were stained with Coomassie blue and scanned with a Hoefer GS300 scanning densitometer to estimate the area under each peak. Relevant protein bands were also excised and, after eluting with 80% methanol at 95 °C, the Coomassie blue stain in each band was quantitated at 595 nm in a spectrophotometer.¹⁰ RBC phospholipids

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Table 1. Hematologic Values for the Two Patients

Variable	C.H.*		B.H.
	Before Splenectomy	After Splenectomy	
Age (yr)	4	6	4
Hemoglobin (g/dL)	9.2	15.2	10.2
Reticulocytes (%)	20	1.4	8.8
MCHC (g/dL)	35.0	35.9	34.6
Microspherocytes (0-4+)	2+	4+	2+

*This patient had hyperbilirubinemia in the newborn period and, before splenectomy, four severe anemic episodes associated with splenomegaly and requiring transfusions. Hematologic values were obtained during stable periods with no recent transfusions. Two normal-appearing siblings have normal hematologic studies.

were assayed using ammonium ferrothiocyanate¹¹ and silica gel (Fischer Silicon Gel G) thin-layer chromatography.¹² Trypsin-Giemsa-banded chromosome studies were performed on phytohemagglutinin-stimulated peripheral blood lymphocytes on two occasions on each family member and on cultures established from skin biopsies of the parents. Multiple blood group and HLA markers were determined on both parents and the two older children.

RESULTS

Hematologic studies. The results of osmotic fragility studies of unincubated RBCs from one affected child (C.H.), are shown in Fig 1. Density centrifugation of fresh peripheral blood enriched the lower or denser, presumably older layers (as judged by the absence of reticulocytes), for spherocytes. Also shown in Fig 1 are the results of osmotic fragility studies obtained at the time of this child's splenectomy. RBCs taken from the splenic pulp and splenic vein had a higher propor-

tion of osmotically fragile RBCs than did the peripheral blood, consistent with splenic "conditioning."^{13,14}

Genetic studies. Metaphase cells from both affected children had an interstitial deletion of the short arm of chromosome 8 extending from band p11.1 to p21.1 (Fig 2): [46,XX,del(8)(p11.1p21.1)]. Peripheral blood lymphocytes from one unaffected sibling and both lymphocyte and skin fibroblast cultures from the parents had normal chromosomal complements.

RBC GSSG-R levels in the affected children were slightly lower than those of either parent, one unaffected sibling, or controls (C.H.: 8.6, 6.3; B.H.: 9.0, 11.2; unaffected sibling: 13.7; mother: 12.0, 15.2; father: 12.4; and nine controls: 13.2 ± 2.4 mmol/min/g Hb SD). Carbonic anhydrase I and II and plasma factor VII levels were not reduced. Blood group and lymphocyte HLA studies gave no evidence of nonpaternity.

Membrane studies. Ektacytometric studies of peripheral blood taken from both parents and the affected children were normal. These findings are consistent with the results noted in patients with classical hereditary spherocytosis in whom no specific skeletal protein defect can yet be identified.⁴

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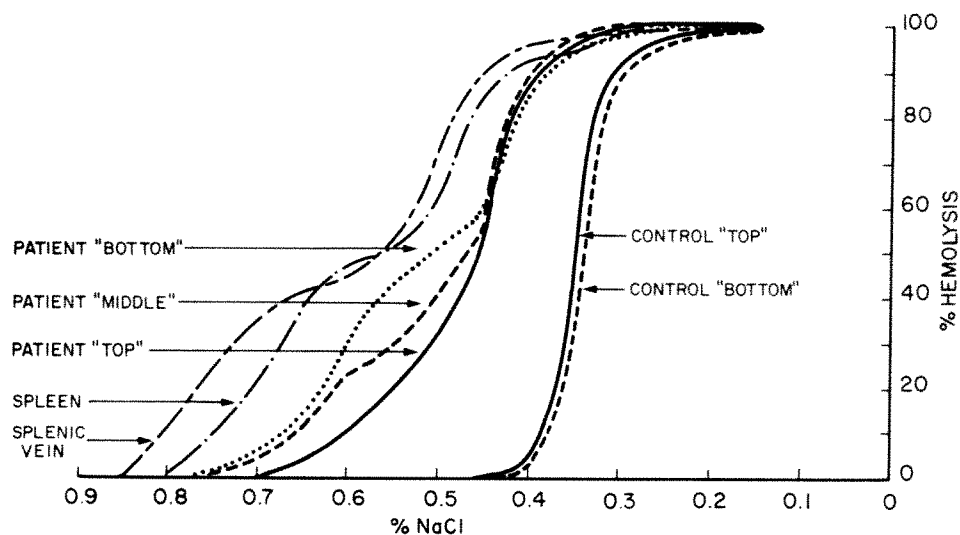


Fig 1. Osmotic fragility studies of C.H.'s peripheral RBCs (separated by density) and splenic RBCs. The results of studies of peripheral blood taken during a routine visit and separated by RBC density (centrifuged at 30 °C for 60 minutes at 27,000 g (ref. 7) show that the least dense fraction with the highest proportion of reticulocytes from the top of the gradient was, as judged by osmotic fragility, the least severely damaged, whereas the denser layers (middle and bottom of the gradient, no reticulocytes) had a higher proportion of spherocytic cells (tail of osmotically fragile cells), suggesting that the patient's RBCs sphered with age. Studies of peripheral blood from a normal control (right-hand curves) showed no shift in osmotic fragility between top and bottom RBC layers. At the time of splenectomy, samples obtained from the splenic pulp and splenic vein contained the highest proportion of spherocytes, suggesting that the spleen was damaging RBCs in transit.

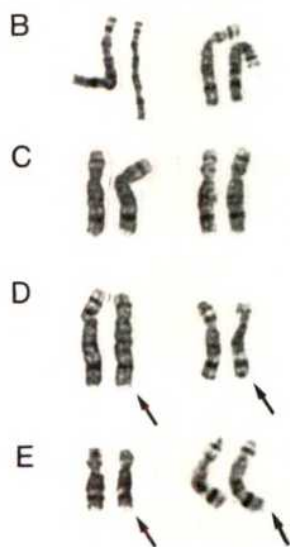
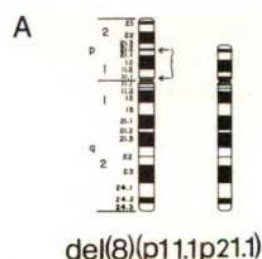


Fig 2. Interstitial deletion of chromosome 8. A: Schematic diagram of the banding pattern of the normal (left) and deleted (right) chromosome 8. The deleted segment is identified by arrows on the normal homologue. B through E: Partial karyotypes of family members illustrating chromosome 8 homologues from trypsin-Giemsa-banded metaphase cells derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. B, father; C, mother; D, patient C.H.; E, patient B.H. D and E: The deleted chromosome is shown on the right of each pair of homologues (arrows).

DISCUSSION

The association of congenital spherocytic anemia in these two siblings with an interstitial deletion of the short arm of chromosome 8 and the absence of these findings in other family members with normal chromosomal complements strongly suggests that the loss of this chromosomal segment resulted in the RBC membrane abnormality. The clinical

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GSSG-R levels were slightly reduced in the two affected children relative to their parents and the unaffected sibling but did not approach half-normal values expected from GSSG-R gene dosage studies in patients trisomic for this region of chromosome 8.¹⁵ These results are ambiguous and do not confirm or deny the cytogenetic findings, which indicate that the deletion extends to 8p21, the region to which the GSSG-R gene has been assigned.¹⁶ It is unlikely that the moderate reduction in GSSG-R activity would cause hemolysis and, in a previous study of a kindred with both HS and GSSG-R deficiency, the two genes segregated independently and family members who had GSSG-R deficiency alone were asymptomatic.¹⁷ Levels of carbonic anhydrase II and factor VII, genes with structural or control loci assigned to chromosome 8,¹⁸ were not reduced, but the deletion observed in our patients probably involved a number of other genes, resulting in dysmorphology and the neurologic findings.

The pattern of inheritance in this family is unusual in that two siblings were affected whereas the parents and a third sibling were chromosomally normal. Cytogenetic analysis on a fourth sibling has not yet been performed, but the child has a normal physical exam and hemoglobin values. Studies of blood groups and HLA antigens in this family were consistent with the presumed paternity. Although other possible mechanisms may explain our observations, the most likely explanation is that one parent is mosaic for this abnormality.

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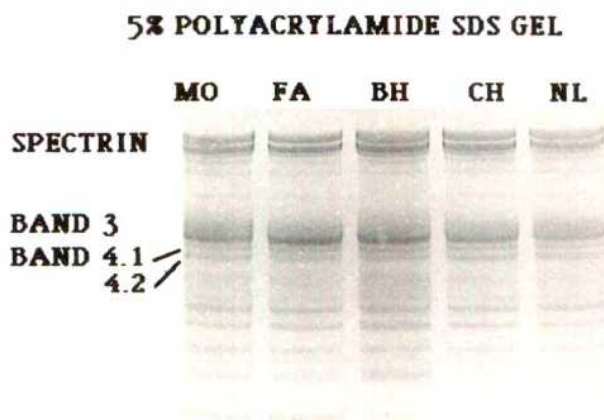


Fig 3. Sodium dodecyl sulfate 3.5% to 7.5% gradient polyacrylamide gel electrophoresis of RBC ghosts. Gels were stained with Coomassie blue. Shown are the gels of both parents (lanes 1 and 2), affected patients C.H. and B.H. (lanes 3 and 4), and a normal control (lane 5). There were no major differences in membrane protein composition between the affected children, parents, and control.

Table 1. Hematologic Values for the Two Patients

Variable	C.H.*		B.H.
	Before Splenectomy	After Splenectomy	
Age (yr)	4	6	4
Hemoglobin (g/dL)	9.2	15.2	10.2
Reticulocytes (%)	20	1.4	8.8
MCHC (g/dL)	35.0	35.9	34.6
Microspherocytes (0-4+)	2+	4+	2+

*This patient had hyperbilirubinemia in the newborn period and, before splenectomy, four severe anemic episodes associated with splenomegaly and requiring transfusions. Hematologic values were obtained during stable periods with no recent transfusions. Two normal-appearing siblings have normal hematologic studies.

were assayed using ammonium ferrothiocyanate¹¹ and silica gel (Fischer Silicon Gel G) thin-layer chromatography.¹² Trypsin-Giemsa-banded chromosome studies were performed on phytohemagglutinin-stimulated peripheral blood lymphocytes on two occasions on each family member and on cultures established from skin biopsies of the parents. Multiple blood group and HLA markers were determined on both parents and the two older children.

RESULTS

Hematologic studies. The results of osmotic fragility studies of unincubated RBCs from one affected child (C.H.), are shown in Fig 1. Density centrifugation of fresh peripheral blood enriched the lower or denser, presumably older layers (as judged by the absence of reticulocytes), for spherocytes. Also shown in Fig 1 are the results of osmotic fragility studies obtained at the time of this child's splenectomy. RBCs taken from the splenic pulp and splenic vein had a higher propor-

tion of osmotically fragile RBCs than did the peripheral blood, consistent with splenic "conditioning."^{13,14}

Genetic studies. Metaphase cells from both affected children had an interstitial deletion of the short arm of chromosome 8 extending from band p11.1 to p21.1 (Fig 2): [46,XX,del(8)(p11.1p21.1)]. Peripheral blood lymphocytes from one unaffected sibling and both lymphocyte and skin fibroblast cultures from the parents had normal chromosomal complements.

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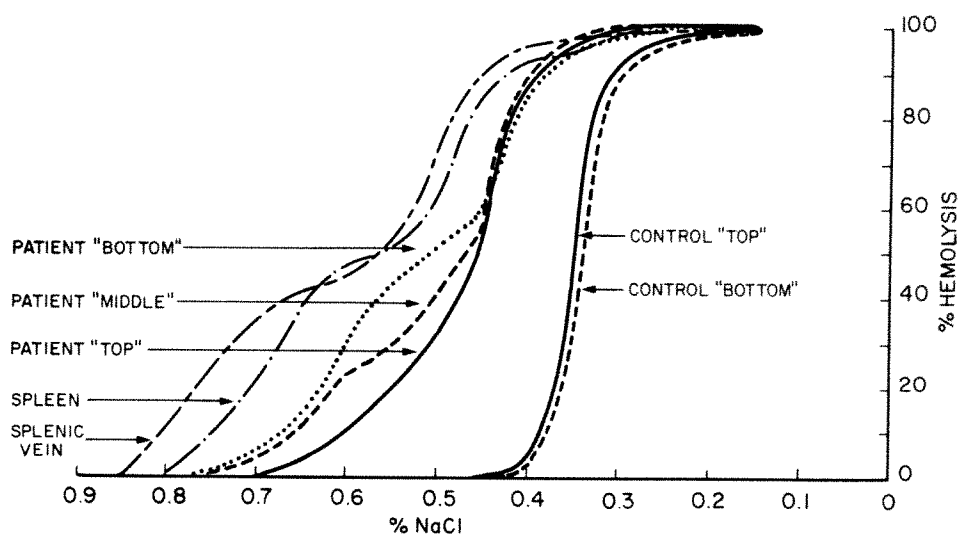


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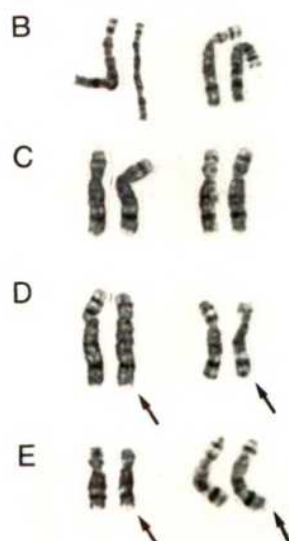
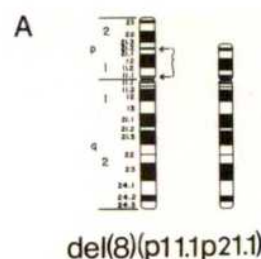


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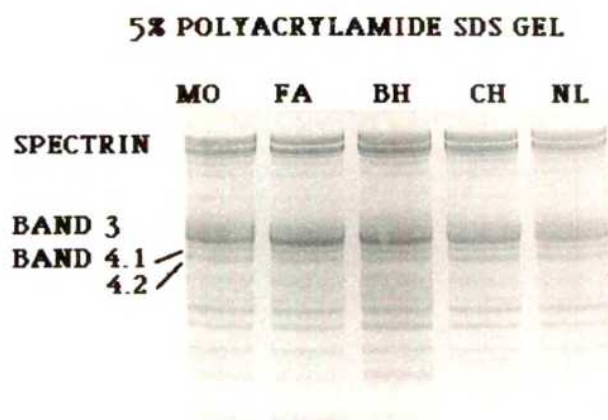


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the results of SDS-PAGE of RBC ghosts derived from peripheral blood of HS patients, as in our two cases, show no qualitative abnormalities.²⁰ Functional studies have detected abnormalities of spectrin-band 4.1 binding²¹ and cytoskeletal structure²² in small groups of patients.

Hemizyosity for a gene on 8p might alter the quantity or function of a membrane protein in a manner not detectable by the semiquantitative gel systems used in this study. For example, an enzymatic deficiency resulting in a failure to modify appropriately a structural protein after translation could affect that protein's binding capacity. Additional studies will be needed to delineate the defect, but these findings

may narrow the chromosomal region in which to search for a gene responsible for spherocytosis; the availability of a cell line* should help in these investigations.

ACKNOWLEDGMENT

We would like to thank Drs Louis Miceli and Edward Klimek for referring the patient and Dr Ted Steck for helpful discussions. Barbara Jones provided technical assistance, and the carbonic anhydrase studies were performed by Dr Richard Tashian.

*A lymphoblastoid cell line from patient B.H. has been placed with the NIGMS Human Genetic Cell Repository, Camden, NJ.

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Selective Expression of *fos* Proto-oncogene in Human Acute Myelomonocytic and Monocytic Leukemias: A Molecular Marker of Terminal Differentiation

By F. Mavilio, U. Testa, N.M. Sposi, M. Petrini, E. Pelosi, C. Bordignon, S. Amadori, F. Mandelli, and C. Peschle

Expression of human *fos* proto-oncogene (*c-fos*) was analyzed in primary cells from 50 untreated acute lymphocytic (ALL) and myeloblastic (AML) leukemias. *c-fos* RNA, analyzed by blot hybridization, was detected virtually only in myelomonocytic (M4) and monocytic (M5) AML. Both M4 and M5 samples show a strong positive correlation between the amount of *c-fos* transcripts and the percentage of leukemic cells expressing surface antigens specific

for mature monocytes and macrophages. Normal mature monocytes exhibit a detectable level of *c-fos* RNA, which is virtually unaltered on activation to macrophage differentiation, but is always below that observed in M4 through M5 monocyticlike cells. These data provide evidence that *c-fos* expression is linked to terminal monocyte and macrophage differentiation in normal and leukemic hemopoiesis.

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THE HUMAN *fos* proto-oncogene (*c-fos*) (ie, the cellular homologue of the transforming gene (*v-fos*) of Finkel-Biskis-Jenkins (FBJ) murine osteosarcoma virus¹ is localized on chromosome 14² and codes for a 380 amino acid nuclear protein of yet unknown function.³ In quiescent human and mouse fibroblasts, transient activation of *c-fos* transcription is detected as soon as 15 minutes after the growth stimulus,⁴⁻⁶ thus implying a role for *c-fos* in cell cycle control. On the other hand, expression of *c-fos* is clearly linked to cell differentiation. Thus, it is enhanced in murine hemopoietic cells or leukemic lines induced by colony-stimulating factors,⁷ as well as in human promyelocytic or monoblastic lines induced by phorbol esters.⁸⁻¹⁰ Furthermore, transfection of cloned mouse *c-fos* into F9 teratocarcinoma cells causes expression of both *fos* mRNA and protein, which is correlated to cell differentiation.^{11,12} Finally, abundant accumulation of *c-fos* transcripts and protein has been observed in late gestation extraembryonic tissues of both murine and human origin.¹³⁻¹⁵

We studied *c-fos* expression in primary cells from 50 untreated human lymphocytic (ALL) and myeloblastic (AML) acute leukemias, accurately classified on the basis of both cell morphology and expression of surface antigen markers. We found significant expression of *c-fos* only in myelomonocytic (M4) and monocytic (M5) AML, with a strong positive correlation between the amount of transcripts and the percentage of leukemia cells expressing surface antigens specific for mature monocytes and macrophages. Expression of *c-fos* was detected even in normal peripheral

blood monocytes, although at a level approximately threefold lower than in leukemic cells.

METHODS

Patients and cells. We selected 50 hematology-oncology patients with acute leukemia of either myelogenous or lymphocytic type. Differential diagnosis of ALL in pre-B, B or T type and of AML in M1 through M5 type was carried out by both morphological evaluation of bone marrow smears according to French-American-British (FAB) criteria¹⁶ and analysis of surface antigen pattern of leukemic cells. All patients were advised of all procedures and attendant risks, and gave informed consent.

Leukemic cells were obtained immediately after diagnosis and before initiation of therapy from peripheral blood or bone marrow aspirates, and enriched up to 90% by centrifugation on Ficoll-Hypaque cushions. Binding of monoclonal antibodies (OKT3, T4, T6, T8, T9, T10, T11, M5, B1, and BA-2 from Ortho Diagnostics, Raritan, NJ; MO2 from Coulter Immunology, Hialeah, FL; Tec-HLA-DR, MG1, and M1 from Tecnogenetics, Turin, Italy; Leu-1 and Leu-2 from Becton Dickinson, Mountain View, CA; R-10, which specifically recognizes human glycoprotein A, generously sent by Dr P.A. Edwards; AN-51 which recognizes human platelet glycoprotein I, kindly provided by Dr A.J. McMichael) was determined by indirect immunofluorescence. Leukemic cells (2×10^6), isolated by centrifugation on a Ficoll-Hypaque cushion, were washed three times in Hank's saline solution and incubated for 60 minutes at 4 °C with 200 μ L of an appropriated dilution in Hank's saline solution containing 1 mg/mL of bovine serum albumin (BSA) of the monoclonal antibody. The cells, washed at 4 °C in Hank's saline solution, were then incubated (60 minutes at 4 °C) with fluorescein isothiocyanate (FITC)-labeled F(ab') fragments of immunoadsorbent-purified sheep antibodies against mouse IgGs. After three additional washes, the cells were mounted on slides in 50% glycerol in phosphate-buffered saline (PBS). The proportion of fluorescent cells was scored in incident light on a Leitz standard universal fluorescent microscope, equipped with a set of filters for narrow-band fluorescence.

Mature resting monocytes were obtained from peripheral blood buffy coat of healthy donors by enrichment on a Ficoll-Hypaque cushion and subsequent purification (>90%) by either centrifugation on a Percoll density gradient¹⁷ or standard adherence on tissue culture flasks.

RNA analysis. Total cellular RNA was extracted from 10^8 cells by the guanidine thiocyanate technique,¹⁸ dotted in 8.0 to 0.5 μ g aliquots onto nitrocellulose paper (BA-85, Schleicher & Schull, Keene, NH), and hybridized¹⁹ to 10^7 cpm of the 1.1-kilobase (kb) *Pst*I/*Pst*I fragment containing most of *v-fos*²⁰, labeled by nick-translation up to a specific activity of 4 to 9×10^8 dpm/ μ g. Stringency of washing (50 mmol/L of NaCl, 5 mmol/L of Na-citrate at 65 °C) was accurately tested on appropriate negative

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controls to avoid nonspecific binding or cross-hybridization of the viral probe. Hybridization to a chicken β -actin probe²¹ was carried out to allow normalization of RNA levels. Poly-A⁺ RNAs were obtained by one passage on oligo-dT cellulose columns, run in 5- μ g aliquots on 1% agarose-formaldehyde gels,²² transferred onto nitrocellulose paper by capillary blot, and hybridized as described for dot blots.

DNA analysis. High mol wt DNA was extracted from 10^8 cells, digested with restriction endonucleases, run on 1% agarose gels, transferred onto nitrocellulose paper, and hybridized to 2×10^7 cpm of radiolabeled probe as described.²³

RESULTS

We analyzed a sample of 20 ALL (8 T ALL, 1 B cell precursor, 8 pre-B, and 3 B ALL) and 30 AML (3 M1, 6 M2, 5 M3, 8 M4 and 8 M5 AML). The expression of *c-fos* in purified leukemic cells was quantitatively evaluated by filter hybridization of total RNA to a *fos*-specific probe (Fig 1). *c-fos* transcripts were barely detectable or undetectable in all leukemic samples, with the exception of myelomonocytic (M4) and monocytic (M5) AML cells, which showed variable levels of *fos* RNA (Fig 1). Analysis of poly-A⁺ RNA by Northern blot (Fig 2) indicates that *c-fos* is transcribed into a predominant 2.3-kb mRNA band and a minor 3.6-kb RNA species, probably a precursor. A faint band of 1.9 kb is also detectable, which apparently corresponds to *fos*-related sequences (*r-fos*), transcribed together with *c-fos* after platelet-derived growth factor (PDGF) stimulation of cultured fibroblasts.²⁴ *c-fos* is thus specifically expressed in M4 and M5 AML, both characterized by presence of a variable proportion of neoplastic cells, more or less differentiated along the monocytic-macrophagic pathway. In none of these cases did we observe amplification of the *fos* gene, as evaluated by Southern blot hybridization of genomic DNA (results not shown).

We further investigated whether in M4 and M5 AML samples expression of *c-fos* was related to the number of monocyticlike cells and/or their stage of differentiation. The former parameter was evaluated on the basis of morphological and cytochemical features (ie, inhibition of α -naphthyl acetate esterase staining after treatment with sodium fluoride, which is specific for monocytes); these also allowed a more precise classification of the M5 AML in M5A and M5B subtypes according to FAB criteria.^{16,25,26} On the other hand, the stage of differentiation of leukemic cells within the monocytic pathway was evaluated by immunofluorescence studies with a panel of ten monoclonal antibodies. In particular, we used OKM5, MO2, and Tec-M1, which recognize surface antigens selectively expressed on mature monocytes and macrophages,²⁷⁻²⁹ as well as on terminally differentiated leukemic lines induced in vitro by various agents.²⁹ All morphological, cytochemical, and immunologic data are summarized in Table 1.

The levels of *c-fos* RNA, showing a virtually equivalent spread in M4 and M5 AML, were not correlated with the number of monocyticlike cells in each sample, as evaluated by standard cytochemical criteria (Fig 1, Table 1). Conversely, a strong positive correlation was observed between the abundance of *c-fos* transcripts and the number of cells recognized by either OKM5, MO2, or Tec-M1 antibodies, in

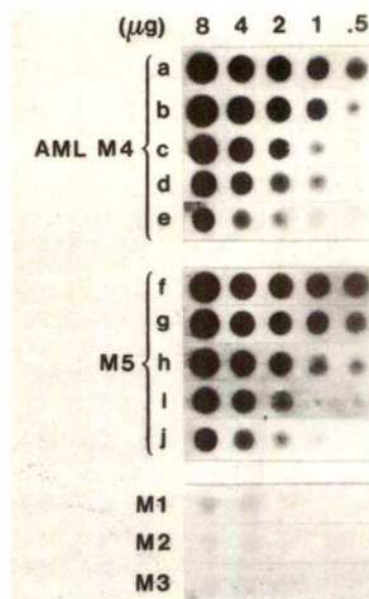


Fig 1. Dot blot analysis of the expression of *c-fos* in human myelomonocytic (M4) and monocytic (M5) acute leukemias (AML), as compared with representative cases of myeloblastic (M1 and M2) and promyelocytic (M3) AML. Total RNA was dotted onto nitrocellulose paper and hybridized to a *v-fos* probe (described in the Materials and Methods section). Autoradiograms were scanned by densitometry, and the values obtained are indicated in Table 1 and plotted in Fig 3. Letters a through j identify the following cases in Table 1: a = MA-82; b = AS-3; c = TO-190; d = LE-104; e = CR-144; f = PR-132; g = DSL-113; h = BA-208; i = VA-134; and j = UMC-122.

both M4 and M5 samples (Fig 3). Among M5 AML, *c-fos* transcripts were more abundant in the M5B subtype (Table 1), which is characterized by a more elevated number of cells morphologically resembling mature monocytes, according to the FAB criteria.

We further investigated *c-fos* expression in normal monocytes. *c-fos* transcripts were clearly detected in nonadherent resting monocytes isolated by centrifugation on density gra-

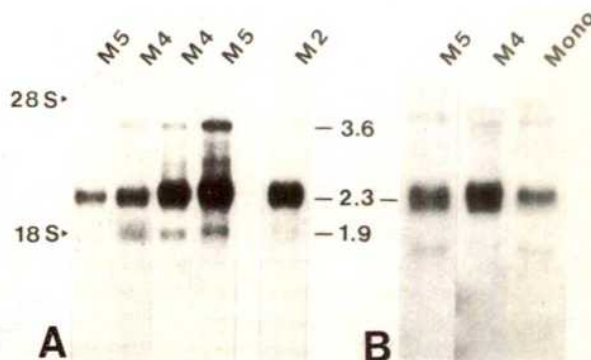


Fig 2. (A) Northern blot analysis of *c-fos* expression in poly-A⁺ RNA obtained from M4 or M5 AML cells and a single case of M2 AML (70% positive to the monocytic-specific MO2 monoclonal antibody, described in text). (B) Northern blot analysis of *c-fos* expression in total cellular RNA from two cases of M4 and M5 AML, as compared with peripheral blood adherent monocytes. Sizes are in kilobases.

Table 1. Surface Antigen Pattern, Cytochemistry, and Expression of *c-fos* in Leukemic Cells From M4 and M5 AML Patients

Case	FAB	Immunofluorescence (% Positive Cells)										Cytochemistry (% Positive Cells)			<i>c-fos</i> Expression (% of Maximum)
		HLA-DR	MG1	OKM5	MO2	M1	OKT9	OKT10	OKT11	R-10	AN-51	Peroxidase	NASDA	NASDA-F	
CR-2	M4	21	81	15	13	ND	Neg	5	Neg	0.2	Neg	35	90	55	39
AS-3	M4	85	66	72	41	ND	Neg	Neg	Neg	Neg	Neg	40	90	55	50
BF-71	M4	ND	ND	9	Neg	Neg	Neg	ND	ND	ND	ND	45	60	60	<1
MA-82	M4	91	89	74	57	69	Neg	ND	ND	Neg	Neg	25	60	60	94
LE-104	M4	34	30	20	0.8	0.8	Neg	ND	ND	Neg	ND	25	70	55	16
DG-145	M4	68	67	69	43	44	4	1.5	Neg	0.1	ND	60	90	60	44
TO-190	M4	25	59	11	5	5.5	0.2	66	ND	Neg	Neg	40	90	40	26
CR-144	M4	ND	ND	5	2	ND	Neg	ND	ND	ND	ND	ND	ND	ND	11
DM-65	M5	ND	51	49	ND	16	Neg	ND	ND	Neg	Neg	Neg	100	0	23
EG-70	M5	53	17	9	2.5	3	Neg	ND	ND	Neg	Neg	Neg	90	30	<1
DSL-113	M5B	28	78	48	42	44	Neg	14	4.5	1	Neg	Neg	80	0	50
UMC-122	M5A	9	51	6	3	3.5	Neg	ND	ND	ND	ND	Neg	80	0	5
PR-132	M5B	75	76	65	40	54	0.8	13	11	Neg	Neg	8	90	10	78
VA-134	M5	11	49	2	ND	1	Neg	Neg	0.2	ND	ND	15	100	0	16
BA-208	M5B	58	79	43	ND	28	Neg	ND	ND	Neg	Neg	Neg	60	0	26
FA-153	M5A	ND	94	2	Neg	Neg	Neg	ND	ND	ND	ND	ND	ND	ND	12

FAB, classification according to the French-American-British criteria.¹⁶ ND, not determined; Neg, negative; NASDA, α naphthyl acetate esterase activity; NASDA-F, NASDA after Na fluoride inhibition.

dient, at a level ~threefold lower than in leukemic cells (as calculated after normalization for the percentage of leukemic cells expressing terminal monocytic markers). On the other hand, monocytes allowed to adhere for 4 hours did not show any detectable variation in *c-fos* expression, as compared with cells before adherence.

We also observed abundant *c-fos* transcription in a single case of M2-AML (Fig 2). These cells were >70% reactive with MO2, even though no other monocytic features could be demonstrated by morphological, cytochemical, and surface antigen criteria.

DISCUSSION

Growing evidence accumulated in the last years suggests a role for "activated" cellular proto-oncogenes (*c-onc*) in establishment, maintenance and progression of neoplastic growth, particularly in the hemolymphopoietic system.³⁰⁻³³ In this regard, abnormal *c-onc* expression, at both quantitative and qualitative levels is significantly associated to different types of human leukemias and lymphomas: however, cause-effect relationship(s) between these abnormalities and oncogenesis of hematopoietic tumors have not been conclusively established so far.³⁴

Previous studies on *c-onc* expression in leukemias have met with various limitations. A crucial aspect is that most observations have been carried out on leukemic lines, which do not often represent faithfully both the genotype and phenotype of the original neoplastic clone(s).³⁵ On the other hand, interpretation of the limited number of studies carried out on primary cells is hampered by both the small number of examined samples and the poor classification of leukemic cells.^{36,37}

We studied the expression of *c-fos* in primary cells from both lymphocytic and myeloblastic acute leukemia patients. Our data indicate that accumulation of *c-fos* transcripts is limited virtually to the myelomonocytic and monocytic sub-

types. The amount of *c-fos* transcripts appears to be strongly correlated with the number of cells expressing surface markers specific for mature monocytes and macrophages, whereas no correlation exists with the number of monocyticlike cells recognized by conventional cytochemical analysis. We interpret these findings to suggest that leukemic cells expressing surface antigens specific for terminal monocytic differentiation selectively accumulate *c-fos* transcripts (ie, the amount of *fos* RNA detected by blot hybridization in the overall sample depends essentially on the relative number of these cells). Therefore, expression of *c-fos* does not generally occur in leukemic cells belonging to the monocytic lineage (eg, monoblasticlike or promonocyticlike elements), but only in the more differentiated ones, as defined by expression of specific surface markers. Accordingly, *c-fos* transcripts in M5 AML are particularly abundant in M5B samples, which are characterized by the presence of a more elevated number of cells resembling mature monocytes at morphological and cytochemical level (Table 1).

Interestingly, a single case of M2-AML abundantly expressed *c-fos* RNA. These cells were strongly positive to one of the monocyte-specific antibodies (ie, MO-2), even though no other monocytic feature could be demonstrated by morphological, cytochemical or surface antigen criteria. This confirms that *c-fos* expression is linked to the genetic program of terminal monocyte differentiation: occasionally, this program may be partially expressed by leukemic cells otherwise differentiated into a nonmonocytic pathway, as a typical example of lineage infidelity.³⁸

Our results also reflect on previous studies on HL-60 (promyelocytic) and U937 (monoblastic) human leukemic lines, which indicated that addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) triggers early *c-fos* transcription and differentiation to adherent macrophages.^{9,10} Interpretation of these studies is rendered uncertain by observations reporting that TPA (as well as other activators of protein kinase C) also induces *c-fos* transcription in various hemo-

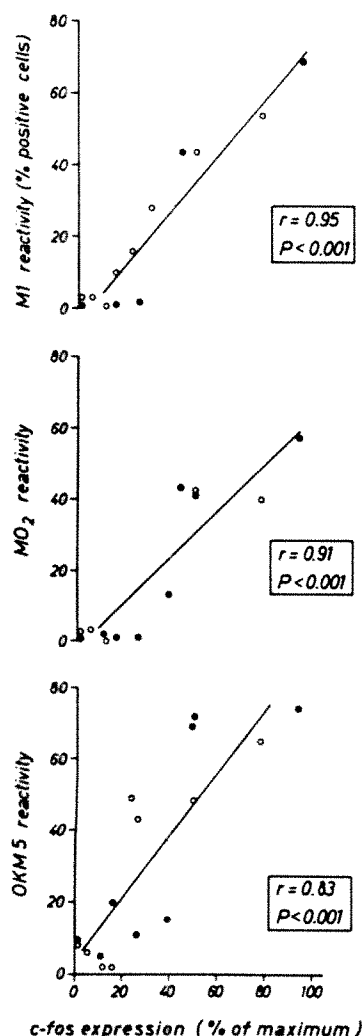


Fig 3. Correlation between expression of *c-fos* (percentage of maximum) and reactivity to monoclonal antibodies OKM5, MO2, and Tec-M1 (percentage of positive cells) in M4 (solid circles) and M5 (open circles) AML cases. The amount of *c-fos* transcript was evaluated by dot blot analysis at total RNA (Fig 1). Reactivity to monoclonal antibodies was assessed by indirect immunofluorescence (described in Materials and Methods Section).

poietic-nonmonocytic or nonhemopoietic lines.^{39,40} Our results strengthen the conclusion that the enhanced *c-fos* transcription in TPA-treated HL-60 and U937 lines may be linked, at least in part, to the induction of terminal monocytic and macrophage differentiation.

A key aspect emerging from our observations is that *c-fos* transcripts are accumulated in normal peripheral blood monocytic cells. We show that the accumulation of *c-fos* transcripts is physiologically linked to terminal differentiation of normal monocytes and is further maintained when they mature to macrophages. Leukemic monocytic cells accumulate *c-fos* transcripts at a level more elevated than that observed in their normal counterparts, however (Fig 2). This discrepancy may hardly be attributed to the more elevated cycling activity of leukemic v normal cells, since M4 through M5 AML elements are mostly blocked in the G0

through G1 through phase of cell cycle.⁴¹⁻⁴³ Indeed, expression of *c-myc*, generally correlated to proliferative activity, is barely detectable in M4 through M5 cells, whereas it is clearly demonstrable in other AML subtypes (M1, M2, M3) showing more intensive proliferation (results not shown here).

In conclusion, our studies provide evidence that *c-fos* transcripts are selectively accumulated in leukemic cells characterized by expression of antigen markers specific for normal mature monocytes and macrophages. In this respect, *c-fos* expression in M4 through M5 AML cells mimics that observed in their normal hemopoietic counterparts, but at a more elevated level. Therefore, expression of *c-fos* may be considered a molecular marker of monocytic differentiation in leukemic cells, possibly of diagnostic significance. Further studies are necessary to assess whether the apparently enhanced expression of *fos* gene in M4 through M5 cells plays a significant role in the leukemogenic process.

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Canine Pluripotent Hematopoietic Stem Cells and CFU-GM Express Ia-like Antigens as Recognized by Two Different Class II-Specific Monoclonal Antibodies

By Friedrich Schuening, Rainer Storb, Sondra Goehle, Joey Meyer,
Theodore Graham, H. Joachim Deeg, and John Pesando

A previous study showed failure of autologous engraftment in lethally irradiated dogs when marrow was treated before infusion with anti-class II antibody 7.2 and complement. The current study extended this observation to a second monoclonal antibody (HB10a) that identifies a different determinant on Ia-like molecules. These results suggest the presence of Ia-like antigens on pluripotent hematopoietic stem cells or on "accessory cells" needed for sustained engraftment to occur. To distinguish between these two possibilities, stem cell-depleted Ia-positive peripheral blood leukocytes obtained by discontinuous albumin density gradient were added as probable source of accessory cells to the marrow inoculum that was depleted of Ia-positive cells by treatment with antibody 7.2 and complement. Eight of ten dogs failed to show engraftment, providing further support for the hypothesis that

pluripotent stem cells and not accessory cells were affected by cytolytic treatment. To provide direct evidence for the presence of Ia-like antigens on canine pluripotent hematopoietic stem cells, autologous transplants were performed using 0.7 to 13×10^6 Ia (7.2)-positive marrow cells per kg obtained with the help of fluorescence-activated cell sorter. Of three evaluable dogs, two showed sustained and complete engraftment, indicating that Ia-like antigens, as recognized by anti-class II antibody 7.2, are expressed at least on part of canine pluripotent hematopoietic stem cells. Concurrent in vitro studies revealed that canine CFU-GM also express Ia-like antigens as recognized by the class II-specific monoclonal antibodies 7.2 and HB10a.

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THE DLA-D region in dogs is thought to be analogous to the I region of the murine major histocompatibility complex and to the HLA-D region in man. In both mouse and man, these regions code for polymorphic bimolecular glycoprotein complexes with molecular weight of approximately 29,000 and 34,000 dalton.¹ There is evidence for the expression of Ia-like antigens in the dog consisting of bimolecular glycoprotein complexes similar to those described in man and mouse.² Ia-like antigens play a crucial role in mediating the cell interactions required in the generation of the immune response.³ Murine and human Ia-like antigens are expressed on B cells, monocytes, and activated T cells. The distribution of Ia-like antigens in dogs appears to differ from that reported for mouse and man in that they are expressed not only on B lymphocytes, monocytes, and activated T cells, but also on most unstimulated T cells.¹

Expression of Ia-like antigens has been demonstrated on human committed hematopoietic precursor cells BFU-E and CFU-GM.^{9,10,25} Controversial results have been published concerning the expression of Ia-like antigens on human

CFU-E.^{8,10,16,21,23-25,27} For lack of an assay for human pluripotent hematopoietic stem cells, it is not presently possible to determine whether Ia-like antigens are expressed on stem cells required for engraftment. Using an in vitro assay for multipotential precursor cells (CFU-GEMM) that represent primitive precursors of multiple lines of hematopoietic differentiation, several groups of investigators have shown that Ia-like antigens are expressed on these very immature hematopoietic precursor cells.^{8,11,17,25} Controversial results have been published concerning the expression of Ia-like antigens on those immature precursor cells responsible for generation of committed progenitor cells in liquid long-term hematopoietic cell cultures.^{18,53-55} Three genetic loci coding for distinct Ia-like molecules have been identified in man, DR, DP, and DQ locus. While HLA-DR and DP antigens have been detected on human CFU-GM, BFU-E, CFU-meg, CFU-GEMM, and, by some investigators, HLA-DR antigens on progenitor cells in long-term cultures, HLA-DQ antigens appear not to be expressed on these hematopoietic progenitor cells.^{8,16,55-58}

The function of Ia-like antigens on hematopoietic precursor cells is unknown, although there is evidence that they may be involved in mediating suppression and/or stimulation of these cells.^{20,24,28} Our previous studies have suggested but not unequivocally demonstrated the presence of Ia-like antigens on canine pluripotent hematopoietic stem cells, as demonstrated by failure of autologous engraftment in lethally irradiated dogs when marrow was treated before infusion with monoclonal anti-class II antibody 7.2 and rabbit complement.²⁹ This result could be due either to the presence of Ia-like antigens on pluripotent hematopoietic stem cells or on accessory cells required for sustained stem cell engraftment to occur.

The present study was aimed at distinguishing between these two possibilities by using "addback" of Ia-positive stem cell-depleted peripheral blood leukocytes to Ia-negative marrow cells and by carrying out grafts with positively selected Ia-positive marrow cells. We also extended our

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initial observation by using a different monoclonal antibody, HB10a, directed against a second epitope on Ia-like antigens. Finally, we studied the expression of Ia-like antigens on committed hematopoietic precursor cells giving rise to CFU-GM.

MATERIALS AND METHODS

Dogs. Beagles, hounds, and mongrels raised at the Fred Hutchinson Cancer Research Center or purchased from commercial kennels were dewormed, vaccinated against distemper, leptospirosis, hepatitis, and parvovirus, and observed for disease for at least 2 months. The dogs weighed 7.5 to 14.3 (median 10.0) kg and were 7 to 17 (median 10.3) months old at the time of transplantation. Research was conducted according to the principles enunciated in the "Guide for Laboratory Animal Facilities and Care" prepared by the National Academy of Sciences—National Research Council.

Monoclonal antibodies (MAbs). MAb 9.6 (IgG2b)³⁰ and MAb 7.2 (IgG2b)³¹ were kindly provided by Dr Paul Martin of this institution. MAb 9.6 does not cross react with canine cells¹ and was used as the negative control antibody in immunofluorescence studies. MAb 7.2 reacts with HLA-DR antigens and cross reacts with Ia-like antigens on canine cells (Fig 1).⁵² MAb HB10a (IgG2a),³² provided by Dr Edward Clark (University of Washington, Seattle), recognizes HLA-DR and -DP antigens⁵³ and cross reacts with canine Ia-like antigens (Fig 1).

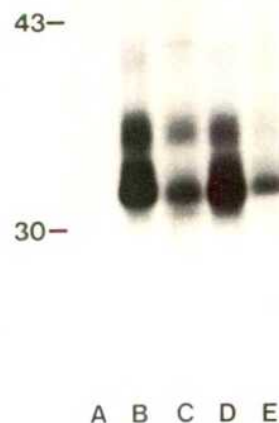
Identification of canine Ia-like antigens. Canine peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Cells were then cultured in Waymouth's medium supplemented with Concanavalin A (20 µg/mL) and 10% canine serum for 48 hours in a humidified 7% CO₂ atmosphere. Washed cells had a viability of greater than 90% and were suspended in phosphate-buffered saline (PBS) at 30 × 10⁶ cells/mL. Cells were surface labeled with ¹²⁵I (1 mCi per 30 × 10⁶ cells) using the lactoperoxidase method as described previously.⁵² Labeled cells were washed in PBS supplemented with 5 mmol/L KI and lysed with Tris-HCl buffered Triton X-100. Nuclei were pelleted by centrifugation and the lysate applied to a Sephadex G-25 column to isolate labeled proteins. Lysates were precleared by incubation with Protein A-Sepharose followed by centrifugation. Immune precipitation was performed by incubating lysate obtained from approximately 1 × 10⁶ cells with additional Protein A-Sepharose that had been incubated overnight with the appropriate mAb. Radiolabeled samples were eluted from washed pellets by heating for three minutes at 95 °C in reducing sample buffer. Antigens were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% to 15% gradient slab gel and visualized by autoradiography.

Total body irradiation, marrow infusion, and postgrafting care. Bone marrow was harvested from anesthetized dogs as previously described³³ and treated as described below. After marrow aspiration, dogs were given 9.2 Gy of total body irradiation at 7 cGy/min from two opposing ⁶⁰Co sources.³⁴ In vitro treated bone marrow was infused within 2 to 14 hours of irradiation. Postgrafting parenteral fluids, electrolytes, platelet transfusions, and antibiotics were administered as indicated.³³ All blood products used for transfusions were irradiated in vitro (15 Gy) to inactivate pluripotent stem cells and immunologically competent cells. Hematocrit, leukocyte, platelet, and differential counts were obtained daily after transplantation. Autopsies with histological examinations were performed on all dogs that died.

Cytolytic treatment of bone marrow. Ia-positive bone marrow cells were eliminated by treating the marrow with mAb and complement (C') as follows:

Bone marrow was diluted 1:3 with Waymouth's medium and

Fig 1. Identification of iodinated Ia-like antigens on canine mononuclear cells by immune precipitation and SDS-PAGE. Characteristic heterodimeric antigens were identified by the HLA class II-specific mAbs used in these experiments. The following mAbs were assayed: A, J-5 (anti-CALLA); B, ISCR3; C, 7.2; D, 9-49; and E, HB10a. Molecular weight standards consist of ¹⁴C-labeled ovalbumin (43 kd) and carbonic anhydrase (30 kd) purchased from New England Nuclear.



separated by density gradient centrifugation over Ficoll-Hypaque (density 1.077) at 1,000 g for 30 minutes. Cells at the interface were washed three times, resuspended to 1 × 10⁶ cells/mL in Waymouth's medium, and incubated with Protein A-Sepharose-purified mAb 7.2 or HB10a (1 mg/10⁹ cells) at room temperature for 60 minutes. The cells were then pelleted and the supernatant was stored at -20 °C for later testing for residual antibody activity (see below). After three further washes, the cells were incubated with undiluted rabbit C' of prescreened batches (absorbed for DR typing, Pelfreeze, Rogers, AR; 1 × 10⁹ cells/50 mL) at room temperature for 60 minutes. The cells were then washed three times and the incubation with C' was repeated. After three washes, the cell pellet was resuspended to 30 mL and infused into the irradiated dog.

Two dogs received marrow cells obtained by density-gradient centrifugation over Ficoll-Hypaque treated twice with C' only as described above but without prior incubation with mAb.

Immunofluorescence studies. In order to determine the efficiency of the cytolytic treatment, bone marrow cells were examined for Ia+ cells before and after incubation with mAb and C' using indirect immunofluorescence and flow microfluorometry. The staining procedure has been described.¹ Briefly, samples of marrow were incubated with mAbs 9.6, 7.2, or HB10a at saturation for 30 minutes at 4 °C. The cells were then washed and incubated with a 1:20 dilution of fluorescein isothiocyanate-conjugated F(ab')₂ fragments of goat antimouse IgG (Tago, Burlingame, Calif). After further washing, cells were analyzed with a fluorescence-activated cell sorter FACS 440 (Becton Dickinson FACS Systems, Sunnyvale, Calif). Positively labeled cells were defined as having fluorescence greater than background fluorescence using mAb 9.6.

Cell sorting. To investigate the characteristics of Ia-positive marrow cells, we collected those cells using a FACS 440 cell sorter. Bone marrow cells obtained by density gradient centrifugation over Ficoll-Hypaque were stained with mAb 9.6, 7.2, or HB10a and fluorescein isothiocyanate-conjugated F(ab')₂ fragments of goat antimouse IgG as described above. Cells were then suspended in Waymouth's medium at a concentration of 5 × 10⁶ cells/mL and sorted into labeled and unlabeled populations on the FACS 440 at a flow rate of 3,000 cells/sec. Fluorescence intensity of the positively sorted cells was greater than that of 99% of cells stained with irrelevant control mAb 9.6. One dog (C108) receiving 7.2 positive autologous marrow cells could not be evaluated because it died in the first week after marrow transplantation from intercurrent infection.

Cell separation by discontinuous albumin density gradient centrifugation. We used a discontinuous albumin density gradient to

deplete or enrich for hematopoietic precursor cells. Peripheral blood lymphocytes and bone marrow cells were separated by density gradient centrifugation over Ficoll-Hypaque. Interface cells were treated with hemolytic buffer (0.155 mol/L ammonium chloride) and separated by a discontinuous albumin density gradient as described by Dicke.³⁵ The stock solution of bovine serum albumin (BSA; 35%) was made using Sigma Fraction V Powder (Sigma Chemical Co., St Louis, Mo). Eighteen mL of a 27% (w/v) BSA solution in Tris buffer were placed in a glass tube (17 × 2 cm) and followed sequentially by 3.75 mL aliquots of solutions containing 25%, 23%, 21%, and 19% BSA. The cell suspension was layered in 9 mL of 17% BSA on top of the gradient. Total cell load per gradient was 0.1 to 1.3 × 10⁹. The osmolality of the Tris buffer and the albumin stock solution was 350 mosmol. The gradient was then centrifuged at 1,000 g for 30 minutes at 10 °C.

CFU-E assay. CFU-E assays were carried out as described.³⁶⁻³⁸ Briefly, bone marrow cells were cultured at a concentration of 2 × 10⁴ cells/0.1 mL plasma clot together with PBL at a concentration of 2 × 10⁴ cells/clot. Cells were incubated for 72 hours in the presence of 1.0 U of erythropoietin (Step III, sheep plasma, Connaught Lab, Willowdale, Canada) per 1 mL of culture medium. Clots were harvested, fixed on glass slides, and stained with benzidine. Aggregates of eight or more hemoglobinized cells were counted as erythroid colonies.

CFU-GM. CFU-GM were carried out as described.³⁹ 0.075 × 10⁶ mononuclear bone marrow cells separated over Ficoll-Hypaque gradient have been cultured per plate for ten days at 37 °C in a humidified 10% CO₂ incubator in 35 mm plastic Petri dishes containing 2 mL of agar medium. The agar medium consisted of an equal volume mixture of 0.6% (w/v) Bacto-agar (Difco, Detroit, Mich) and double-strength Dulbecco's Modified Eagle's Medium (GIBCO, Grand Island, NY) containing 40% (v/v) heat-inactivated human AB plasma. PHA-stimulated lymphocyte-conditioned medium was added to the Petri dishes (0.1 mL/plate) prior to the agar-medium mixture containing the bone marrow cells.

RESULTS

Autologous transplants with marrow treated by mAb 7.2 and C' and added stem cell-depleted Ia-positive peripheral blood leukocytes. We first showed that treatment of marrow with C' alone does not interfere with engraftment (Table 1, dogs C46 and C142). Then, in confirmation of results by others, we determined that peripheral blood leukocytes from fraction 5 of albumin density gradient were depleted of hematopoietic stem cells insofar as they failed to achieve hematopoietic reconstitution after lethal irradiation (Table 1, dog C86). By further characterizing peripheral leukocytes in fraction 5 using differential counts and flow microfluorometry, we showed that 63 ± 11% of the cells are lymphocytes, 4 ± 2% monocytes, 34 ± 11% polymorphonuclear granulocytes, and that 72 ± 18% of the cells reacted with anti-class II mAb 7.2, 78 ± 15% with anti-class II mAb HB10a, and 66 ± 11% with anti-Thy1 mAb F3-20-7 (data represent mean value ± 1 SD of three independent experiments).

Next, 10 dogs received marrow cells treated in vitro with mAb 7.2 and C' before infusion. The number of mononuclear marrow cells infused after in vitro treatment ranged from 0.2 to 2.4 × 10⁸/kg (Table 1). In addition, eight of the 10 dogs received between 0.04 to 0.9 × 10⁸/kg of Ia-positive peripheral blood leukocytes from fraction 5 of albumin density gradient. To increase the number of stem cell-depleted Ia-positive peripheral blood leukocytes, two of the 10 dogs received cells from albumin density gradient fractions 4 and 5 (Table 1, dogs C122 and C115).

In two of the 10 dogs, marrow incubation with mAb 7.2 and C' was carried out at 5 °C (dogs C106 and C107). This

Table 1. Results in Dogs Given 9.2 Gy Total Body Irradiation and Infusion of Autologous Bone Marrow Treated With Complement Only (Group 1), Stem Cell-Depleted Ia + Peripheral Blood Leukocytes Only (Group 2), Autologous Bone Marrow-Treated With mAb 7.2 and Complement, and Stem Cell-Depleted Ia + Peripheral Blood Leukocytes (Group 3)

Group/ Dog No.	Incubation Temp	Marrow Cell # Infused ($\times 10^{-8}$ /kg)	7.2 + Cells (%)*		HB10A + Cells (%)*		Cell No. of Fraction 4 + 5 ($\times 10^{-8}$ /kg)	Sustained Graft	Histological Signs of Engraftment at Autopsy	Survival (d)
			Pretreatment	Posttreatment	Pretreatment	Posttreatment				
Group 1										
C46	Room temp	1.3	—	—	—	—	—	Yes	—	>60
C142	Room temp	0.8	—	—	—	—	—	Yes	—	>60
Group 2										
C 86	—	None	—	—	—	—	Frac 5: 0.04	No	No	9
Group 3										
C106	5 °C	2.4	15	4	4	0	Frac 5: 0.9	Yes	—	>60
C107	5 °C	0.6	11	14	14	14	Frac 5: 0.1	Yes	—	>60
C110	Room temp	0.5	11	0	15	0	Frac 5: 0.06	No	No	8
C114	Room temp	0.16	7	0	6	0	Frac 5: 0.07	No	No	18
C126	Room temp	0.4	16	0	17	0	Frac 5: 0.12	No	No	11
C111	Room temp	0.2	28	2	43	0	Frac 5: 0.10	No	No	11
C45	Room temp	0.4	9	0	13	0	Frac 5: 0.04	No	No	12
C58	Room temp	0.4	35	0	12	0	Frac 5: 0.14	No	Yes	21
C122	Room temp	0.3	25	2	19	0	Frac 5: 0.2	No	No	14
C115	Room temp	0.8	21	1	5	0	+ Frac 4: 0.3	No	Yes	9
							Frac 5: 0.2			
							+ Frac 4: 0.2			

*Expressed as percentage of a total of 4,000 cells counted by flow microfluorometry whose fluorescence exceeded a gate defining the negative control population (stained with irrelevant mAb 9.6), corrected for the percentage of the negative control.

Table 2. Enrichment for Hematopoietic Precursor Cells From Canine Marrow by Albumin Density Gradient*

	CFU-GM (Per 10 ⁵ BMC)†	CFU-E (Per 10 ⁵ BMC)†
Unseparated marrow	106 ± 4	151 ± 59
Fraction 1	9 ± 1	54 ± 25
Fraction 2	41 ± 4	85 ± 17
Fraction 3	2176 ± 227	3275 ± 340
Fraction 4	335 ± 15	211 ± 41
Fraction 5	31 ± 1	5 ± 7

*Data give representative examples of three independent experiments.

†Cultures were plated in triplicate (CFU-GM) or sixfold (CFU-E). Colony numbers represent mean value ± 1 SD.

resulted in incomplete killing of Ia-positive marrow cells as documented by the finding of 7.2 and HB10a-positive cells subsequent to marrow incubation (Table 1). Both dogs showed sustained and complete hematopoietic engraftment. In the remaining eight dogs, marrow was treated at room temperature, which resulted in complete elimination of Ia-positive cells as detected by mAb 7.2 and HB10a in 5 dogs and only 1% to 2% residual Ia-positive cells in 3 dogs as detected by mAb 7.2. These eight dogs failed to show sustained hematopoietic engraftment despite the addition of Ia-positive peripheral blood mononuclear cells. Supernatants from marrow treated with mAb 7.2 and C' were tested for residual antibody by incubating 1×10^6 normal canine peripheral blood mononuclear cells with 100 μ L of the supernatants and then fluorescein isothiocyanate-conjugated F(ab')₂ fragments of goat antimouse IgG. All supernatants reacted with the target cells, confirming that conditions of antibody excess were met.

Autologous transplants with Ia (7.2)-positive marrow cells obtained by fluorescence-activated cell sorting. Additional studies were designed to provide direct evidence for the presence of Ia-like antigens on canine pluripotent stem cells. Marrow cells were separated by Ficoll-Hypaque gradient and then split into fractions using the discontinuous albumin density gradient. A marked enrichment of committed hematopoietic precursor cells, CFU-GM and CFU-E, was seen in

fraction 3 of the gradient (Table 2). Autologous transplants of cells from fraction 3 resulted in prompt, sustained, and complete hematopoietic engraftment (Table 3, dogs C116 and C141). To exclude the possibility, that coating of marrow cells with mAb 7.2 interferes with engraftment, we performed autologous transplants using marrow cells incubated with mAb 7.2 in the absence of C'. Both dogs so treated showed sustained engraftment (Table 3, dogs C124 and C117).

Marrow cells obtained in fraction 3 of the albumin density gradient were then stained at saturation with antibody 7.2 and fluorescein isothiocyanate-conjugated F(ab')₂ fragments of goat antimouse IgG. 7.2-positive cells were selected on the fluorescence-activated cell sorter and infused into lethally irradiated autologous recipient. FACS analysis after cell sort demonstrated that 99% of the positively sorted cells had fluorescence intensity greater than that of cells stained with irrelevant control mAb 9.6. Transplantation results are shown in Table 3, group 3. Dog C134 received 0.007×10^8 cells/kg body weight. This dog showed a rise in white blood cell count on day 9 after marrow transplant, with 60% of the cells being granulocytes. The dog died on day 11 after marrow transplant due to septicemia. Marrow histology at autopsy showed 2% of normal cellularity identifying myeloid as well as erythroid precursor cells. In order to increase the number of 7.2-positive marrow cells transplanted into the dogs, we increased the duration of the cell sort and used two cell sorters simultaneously. As a result dogs C228 and C281 received 0.03 and 0.13×10^8 marrow cells/kg body weight, respectively. Both dogs showed sustained hematopoietic engraftment and are now surviving longer than 210 days after marrow transplant with complete recovery of marrow function. Speed of neutrophil and platelet recovery was prolonged compared to control dogs and varied depending on number of marrow cells transplanted. Dog C228 receiving 0.03×10^8 marrow cells/kg body weight had granulocyte counts above 1,000 granulocytes/ μ L on day 26, compared to a median of 12 days (range 8 to 14 days) in controls.⁵¹ Platelet counts were above 20,000 platelets per μ L on day 52 (median in control dogs day 15, range 11 to 23 days). Dog C281 receiving 0.13×10^8 marrow cells/kg body weight had

Table 3. Autologous Marrow Transplants in Dogs Given 9.2 Gy Total Body Irradiation and Infusion of (Group 1) Fraction 3 of Albumin Density Gradient, (Group 2) Marrow Cells Coated With Monoclonal Antibody 7.2 Without Complement, (Group 3) 7.2 + Marrow Cells Sorted Out With FACS 440

Group/ Dog #	Marrow Cell # Infused ($\times 10^{-8}$ /kg)	Method to Preenrich for Stem Cells Before Cell Sort	Sustained Graft	Histological Signs of Engraftment at Autopsy	Survival (d)
Group 1					
C116	frac 3: 0.3	—	Yes	—	>60
C141	frac 3: 0.25	—	Yes	—	>60
Group 2					
C124	7.2 coated: 1.0	—	Yes	—	>60
C117	7.2 coated: 1.0	—	Yes	—	>60
Group 3*					
C134	7.2+: 0.007	frac 3	No	Yes	11
C228	7.2+: 0.03	frac 3	Yes	—	>210
C281	7.2+: 0.13	frac 3	Yes	—	>210

*Fluorescence intensity of the positively sorted cells was greater than that of 99% of cells stained with irrelevant control mAb 9.6.

>1000 granulocytes/ μ L already on day 16 and >20,000 platelets/ μ L on day 38.

Autologous transplants with bone marrow treated in vitro with mAb HB10a and C'. In an attempt to extend our previous results obtained with mAb 7.2, we performed autologous transplants using marrow treated with a different anti-class II mAb, HB10a, and C'. Both mAbs identify characteristic heterodimeric complexes by immune precipitation on iodinated Ia-positive canine cells (Fig 1). MAb HB10a identifies both HLA-DR and -DP molecules on human cell populations on which mAb 7.2 is HLA-DR specific, indicating that these two mAbs identify different antigenic determinants.⁵² This conclusion is further supported by the observation that these two Ia-specific mAbs differ in their cross reactivity with additional animal species (Ladiges et al, unpublished). It remains to be determined whether or not these two mAbs identify distinct populations of Ia-like molecules in the dog. Four of five dogs failed to show sustained hematopoietic engraftment (Table 4). The fifth dog (C120) had slow recovery of granulocyte and platelet counts after marrow transplantation. Granulocyte counts were above 1,000 granulocytes/uL on day 26, compared to a median of 12 days (range 8 to 14 days) in controls.⁵¹ Platelet counts were above 20,000 platelets/uL on day 42 (median in control dogs, day 15, range 11 to 23 days).

Hematopoietic in vitro studies. Concurrent in vitro studies revealed that treatment of marrow cells with mAb 7.2 and C' as well as with mAb HB10a and C' impaired growth of CFU-GM (Table 5). Expression of Ia-like antigens by CFU-GM was in addition examined by sorting 7.2-positive and -negative marrow cells and plating them in CFU-GM assay. Only 7.2-positive marrow cells showed growth of CFU-GM (Table 5). Similar results were obtained after plating HB10a-positive and -negative marrow cells in CFU-GM (Table 5).

DISCUSSION

We have previously shown that dogs given lethal total body irradiation and an infusion of autologous marrow treated in vitro with anti-class II mAb 7.2 and rabbit C' failed to achieve hematopoietic reconstitution.²⁹ In our present study, we extended these observations with mAb 7.2 to a different anti-class II mAb, HB10a, which recognizes HLA-DR and -DP antigens⁵² and cross reacts with Ia-like antigens on canine cells (Fig 1). These results provide additional

circumstantial evidence favoring the presence of Ia-like antigens on canine pluripotent stem cells.

Another explanation for the failure of engraftment seen with antibody-treated autologous marrow is that Ia-like antigens are not expressed on pluripotent hematopoietic stem cells but rather on "accessory" cells required for stem cells to engraft. We tried to distinguish between these two possibilities by adding stem cell-depleted Ia-positive peripheral blood leukocytes to a marrow inoculum that was depleted of Ia-positive cells, thereby presumably providing accessory cells. As it is possible to achieve complete marrow repopulation after otherwise lethal total body irradiation with peripheral blood cells only, this cell population contains stem cells as well as accessory cells and therefore can be used as a source for accessory cells. However, eight of ten dogs so treated failed to show sustained hematopoietic engraftment. This result provided further support for our hypothesis that Ia-like antigens as detected by anti-class II mAb 7.2 are expressed on canine pluripotent stem cells. The sustained engraftment seen in two of the ten dogs was most likely due to the fact that incubation with antibody and C' was carried out at 5 °C rather than at room temperature, resulting in incomplete elimination of Ia-positive cells from the marrow.

It is possible, of course, that if those dogs receiving Ia-depleted marrow had survived longer, reconstitution of hematopoiesis might have occurred. With existing supportive measures, this possibility cannot presently be investigated. We therefore tried to test the hypothesis of expression of Ia-like antigens on pluripotent canine stem cells directly by carrying out grafts with marrow cells positively selected for Ia-like antigens as detected by anti-class II mAb 7.2 using the fluorescence-activated cell sorter. Of the evaluable three dogs, one had only transient hematopoietic engraftment, most likely due to very low marrow cell numbers infused (7×10^5 /kg). With an increase in the duration of the cell sort, we increased the number of Ia-positive marrow cells transplanted. Two subsequent dogs given 3 and 13×10^6 Ia-positive marrow cells/kg showed sustained and complete hematopoietic engraftment. This result is in keeping with our hypothesis, and provides direct evidence for the expression of Ia-like antigens as detected by anti-class II mAb 7.2 on at least part of pluripotent canine hematopoietic stem cells.

Prendergast et al⁶⁰ recently described complete although slow marrow recovery in a total of 7 out of 10 dogs after lethal TBI and infusion of autologous Ia-depleted marrow using a different anti-class II mAb (WM-2). These results are in contrast to what we have seen using two different

Table 4. Results in Dogs Given 9.2 Gy Total Body Irradiation and Infusion of Autologous Marrow Treated With Monoclonal Antibody HB10a and Complement

Dog #	Marrow Cell # Infused ($\times 10^{-6}$ /kg)	HB10A+ Cells (%)*		7.2+ Cells (%)*		Sustained Graft	Histological Signs of Engraftment at Autopsy	Survival (d)
		Pretreatment	Posttreatment	Pretreatment	Posttreatment			
C173	1.4	ND	ND	ND	ND	No	No	6
B935	1.0	ND	ND	ND	ND	No	No	13
C120	1.7	20	3	13	1	Yes	—	>60
BC 5	0.2	43	3	10	3	No	No	17
C146	0.5	28	0	10	0	No	No	14

*Expressed as percentage of a total of 4,000 cells counted by flow microfluorometry whose fluorescence exceeded a gate defining the negative control population (stained with irrelevant mAb 9.6), corrected for the percentage of the negative control.

Table 5. Effect of Anti-Ia-antibody Plus Complement Treatment on the Growth of CFU-GM of Dog Marrow Cells (Experiment 1): In Vitro Culture Results of Ia+ and Ia- Canine Marrow Cells as Detected by Monoclonal Antibodies 7.2 and HB10a After Cell Sort on FACS 440 (Experiment 2)

	Colony Number After Treatment With*			% Inhibition†	
	C' Only	7.2 + C'	HB10a + C'	7.2 + C'	HB10a + C'
Experiment 1					
CFU-GM (per 10 ⁵ BMC)	116 ± 17	0.4 ± 0.7	0.4 ± 0.7		
	278 ± 114	28 ± 5	5 ± 2	96 ± 4	99 ± 1
	55 ± 6	0.9 ± 0.6	0		
	7.2 + BMC*	7.2 - BMC*	HB10a + BMC*	HB10a - BMC*	
Experiment 2	135 ± 19	0	34 ± 12	0	
CFU-GM (per 10 ⁵ BMC)	96 ± 14	0	—	—	
	56 ± 7	0	—	—	

*Cultures were plated in triplicate. Cell number plated after treatment with mAb + C' was adjusted to cell count after treatment with C' only. Colony numbers represent mean value ± 1 standard deviation.

†Data represent % inhibition of antibody plus complement-treated cells compared to cells treated with complement only.

anticlass II mAbs and may either be due to incomplete elimination of Ia-positive marrow cells or to differential expression of Ia-like antigens as recognized by mAbs 7.2, HB10a, and WM-2 on canine pluripotent hematopoietic stem cells. Efficiency of removal of Ia-positive marrow cells was analyzed by fluorescence microscopy counting 200 cells rather than by fluorescence-activated cell sorter counting 4,000 cells. Engraftment was seen only in those dogs receiving a relatively high mean cell dose of $9.4 \times 10^7/\text{kg}$ compared to those dogs that failed engraftment (mean cell dose $3.5 \times 10^7/\text{kg}$), suggesting that the first group might have received a sufficient number of remaining Ia-positive marrow cells to allow marrow recovery. Unfortunately the authors did not provide data of transplants performed with marrow cells positively selected for Ia-like antigens as detected by mAb WM-2.

Detection of Ia-like antigens on most of human committed hematopoietic precursor cells,^{4-9,12-14,16,19,20,26,27} and the demonstration of Ia-like antigens on human B lymphocytes and monocytes⁴³⁻⁴⁵ as well as on human lymphocytic and myelocytic leukemia cells,^{26,45} have prompted the suggestion that human pluripotent hematopoietic stem cells also express Ia-like antigens. For lack of an assay for human pluripotent hematopoietic stem cells, it is not presently possible to prove this hypothesis directly. An in vitro assay for multipotential progenitor cells (CFU-GEMM, colony-forming units: granulocyte, erythroid, monocyte, megakaryocyte) has been developed.⁴⁶ These human multipotential progenitors may be closely related to murine CFU-S in that they represent primitive precursors of multiple lines of hematopoietic differentiation. Several groups of investigators have shown that Ia-like antigens are expressed on these very immature hematopoietic precursor cells.^{8,11,17,25} Controversial results have been published concerning the expression of Ia-like antigens on those immature precursor cells responsible for proliferation of committed progenitor cells in liquid long-term hematopoietic cell cultures. Moore et al¹⁸ could not identify Ia-like antigens on those progenitor cells in long-term cultures. However, Andrews et al⁵³ found inhibition of hematopoietic precursor cells in a similar long-term culture system after treatment with anti-HLA-DR antibody and C'. Keating et al⁵⁴ could not detect Ia-like antigens on precursor cells responsible for the proliferation of CFU-GM in a different

(one stage) long-term culture system. In all these studies, only depletion techniques were used to identify Ia-like antigens on precursor cells, giving rise to long-term culture systems. Recently, Falkenburg et al⁵⁵ showed the expression of HLA-DR determinants on precursor cells responsible for sustained proliferation of CFU-GEMM, BFU-E, and CFU-GM in liquid long-term cultures, using both fluorescence-activated cell sorting and C'-dependent cytotoxicity assays. Three genetic loci coding for distinct Ia-like molecules have been identified in man, DR, DP, and DQ locus. While HLA-DR and DP antigens have been detected on human CFU-GM, BFU-E, CFU-meg, CFU-GEMM, and, by some investigators, HLA-DR antigens on progenitor cells in long-term cultures, HLA-DQ antigens appeared not to be expressed on these hematopoietic progenitor cells.^{8,16,55-58} However, a recent publication by Sparrow et al describes expression of HLA-DQ antigens on three subpopulations of CFU-GM.⁵⁹

Using the CFU-S assay in mice, Basch et al⁴⁷ have reported that a heteroantiserum against Ia antigens was not reactive with murine CFU-S in cytotoxicity and fluorescence-activated cell sorting studies. They concluded that CFU-S lack Ia antigens. Recent evidence, however, suggests that the situation is more complex. The murine I region is composed of at least five subregions.⁴⁸ Murine Ia antigens encoded by the I-E subregion have amino acid homology with human DR antigens.⁴⁹ Fitch et al⁵⁰ found no effect of monoclonal antibodies against I-A subregion gene products on CFU-S, but potent inhibition of these cells by anti-I-E subregion heteroantibodies and C'. These observations suggest that murine pluripotent hematopoietic stem cells express I-E subregion antigens (equivalent to human HLA-DR) but not I-A subregion antigens (equivalent to human HLA-DQ). Performing autologous marrow transplants in rhesus monkeys, Gerritsen et al²² observed graft failure after incubating the marrow with a combination of anti-RhLA-DR7 antibodies and C', suggesting that the RhLA-DR7 antigen is present on the hematopoietic stem cells of the rhesus monkey. Our previous²⁹ and present results in dogs, showing that Ia-like antigens as detected by anti-class II mAb 7.2 are expressed at least on part of canine pluripotent hematopoietic stem cells, are therefore in agreement with observations reported for human multipotential progenitor cells and for hemato-

poietic stem cells of mice and rhesus monkeys. Most importantly, we showed this to be true not only by methods of negative selection but also by demonstrating complete hematopoietic reconstitution with transplants of cells positively selected for expression of Ia-like antigens as detected by anti-class II mAb 7.2.

Concurrent *in vitro* studies revealed that canine CFU-GM also express Ia-like antigens as recognized by two mAbs (7.2 and HB10a) that identify different determinants on canine Ia-like molecules. We showed this by employing positive and negative selection for the appropriate cells.

Results on canine CFU-GM are in agreement with those reported for man. Both with C'-dependent cytotoxicity assays and fluorescence-activated cell sorting, Ia-like antigens were demonstrated on human CFU-GM using polyclonal and monoclonal antibodies.

Our findings indicate that Ia-like antigens, recognized by anti-class II antibody 7.2, are expressed on most true

pluripotent hematopoietic stem cells in the dog. This seems to be relevant for clinical bone marrow transplantation. Both human and canine lymphoma cells express Ia-like antigens, and transplantation of autologous marrow purged of Ia-positive tumor cells seems an attractive treatment modality. Present results suggest that this may not be safe. At the present time, it is uncertain whether true pluripotent hematopoietic stem cells in man are Ia positive or negative and, if Ia positive, whether they express only HLA-DR but not HLA-DQ, thus allowing selective removal of HLA-DQ-positive neoplastic cells.

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Interaction of Ristocetin and Bovine Plasma With Guinea Pig Megakaryocytes: A Means to Enrich Megakaryocytes Based on Membrane Rather Than Physical Characteristics

By Carl W. Jackson, Shirley A. Steward, Nancy K. Hutson, and Richard A. Ashmun

We have investigated whether megakaryocytes can be aggregated by ristocetin and bovine plasma and whether such aggregation can be used as a step in the purification of megakaryocytes from marrow cell suspensions. Guinea pig marrow cell suspensions were first enriched for megakaryocytes by density equilibrium centrifugation in continuous Percoll density gradients. The megakaryocyte-enriched marrow was stirred in a platelet aggregometer to which ristocetin or bovine plasma was added. Megakaryocytes were aggregated by both ristocetin and bovine plasma with the proportion aggregated being related to the concentration of ristocetin or bovine plasma. Maximal aggregation (>90% of megakaryocytes) was achieved with 2.0 mg/mL ristocetin or 5% bovine plasma and required five minutes. All maturation stages of morphologically recognizable megakaryocytes were aggregated. The megakaryocyte aggregates were separated from the marrow suspension by sedimentation at 1 g and the megakaryocytes disaggregated by dilution with media (ristocetin

aggregated) or addition of dextran sulfate (bovine plasma aggregated). Megakaryocyte purity and recovery were higher with bovine plasma than with ristocetin. A mean of 92% of the megakaryocytes in the bovine plasma aggregated cell suspensions were recovered with megakaryocytes constituting an average of 76% of the final cell suspensions. The viability as well as the diameters and DNA content distribution of these megakaryocytes were similar to those of the starting population. We conclude that guinea pig megakaryocytes behave like platelets in that they can be aggregated with ristocetin or bovine plasma and that megakaryocyte aggregation induced by ristocetin or bovine plasma provides a means to enrich these cells based on membrane rather than physical characteristics. This approach yields purified megakaryocyte populations that are representative of those in unfractionated marrow.

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HIGHLY purified populations of megakaryocytes are required to study many aspects of megakaryocyte differentiation. Most methods of megakaryocyte enrichment employ a sedimentation step in which megakaryocytes are separated from other marrow cells by virtue of their larger size.¹⁻¹¹ Small megakaryocytes that tend to be immature may be selectively lost by such procedures.¹² This problem might be overcome if both large and small megakaryocytes could be specifically aggregated so that all megakaryocytes would sediment together. The observation that bovine von Willebrand factor (vWF)¹³⁻¹⁶ alone and ristocetin¹⁷ in the presence of vWF¹⁸ will aggregate human as well as guinea pig¹⁹ platelets suggested the possibility that megakaryocytes also might be aggregated by these reagents. Platelets so aggregated can be disaggregated if the platelet release reaction is inhibited during aggregation.²⁰ In this study, we have examined whether guinea pig megakaryocytes can be aggregated by bovine plasma and ristocetin and whether such aggregation can be used as a step in the purification of megakaryocytes.

MATERIALS AND METHODS

Young adult male guinea pigs of the Hartley strain were purchased from Hazelton Research Animals (Denver, Pa) or Charles River Breeding Labs (Wilmington, Mass). Ristocetin was purchased from Curtin Matheson Scientific, Inc. (Houston, Tex) and citrated bovine plasma from Hyclone Laboratories (Logan, Utah). Purified bovine von Willebrand factor was a generous gift of Dr E.P. Kirby, Temple University, Philadelphia, Pa. Normal porcine plasma and plasma from a pig with von Willebrand disease were a generous gift of Drs L.A. Solberg and E.J.W. Bowie, Mayo Clinic, Rochester, Minn. Percoll density media was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and autoclaved before use. Fetal calf serum was obtained from GIBCO (Grand Island, NY).

Collection of marrow and preparation of megakaryocyte-enriched marrow cell suspensions by density fractionation. Guinea pigs were killed by exsanguination under ether or sodium thiamy-

lal anesthesia. Blood was collected from the dorsal aorta into 0.1 mmol/L acid citrate and plasma prepared. Marrow from tibias, femurs, and humeri was flushed with 2.5 mL of CATCH media² containing DNase I³ and suspended by gentle pipetting using Pasteur pipettes siliconized with Prosil 28 (PCR, Inc., Gainesville, Fla). Unless indicated otherwise, later references to CATCH media indicate the above formulation. Cell concentration was determined using a hemacytometer. Initial enrichment of megakaryocytes was achieved by equilibrium density centrifugation in Percoll density gradients based on the studies of Rabellino et al⁵ who found that human megakaryocytes had a lower buoyant density than 90% of the other marrow cells. Stock Percoll density media was made isotonic with 10X Hanks' balanced salt solution (BSS) without Mg⁺⁺ and Ca⁺⁺ and diluted to the desired concentration with CATCH media. A 10 mL continuous linear gradient made of 0% to 36% Percoll density media with a gradient density range of 1.02 to 1.05 g/mL was formed on top of 2 mL of 45% Percoll in a 15 mL plastic centrifuge tube. The continuous gradient was constructed using a linear gradient maker with 5 mL of the above pooled marrow suspension in the low density chamber and 5 mL of 36% Percoll in the high density chamber. A continuous rather than a discontinuous density gradient was used so as to minimize contact of megakaryo-

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cytes with each other. The gradient was centrifuged for 20 minutes at 1,000 g at room temperature and the top 10 mL removed including cells in the 36%:45% Percoll interface, diluted 1:1 with CATCH media and the cells pelleted (600 × g). More than 95% of the megakaryocytes were present in this fraction. The supernatant was discarded and the cells resuspended in CATCH media with 12.5 mmol/L Na₂ EDTA¹⁶ to prevent activation (for aggregation studies with bovine plasma) or in CATCH media with various concentrations of fresh, citrated guinea pig plasma plus 5 mmol/L Na₂ EDTA²⁰ and usually 5% fetal calf serum (for ristocetin aggregation).

Aggregation studies with ristocetin and bovine plasma. The megakaryocyte-enriched cell suspension (usually 0.7 mL) was stirred (1,200 rpm) in a platelet aggregometer (Chrono-Log Corp., Havertown, Pa) at 37 °C in a "0.312" diameter siliconized cuvette to which various concentrations of ristocetin or bovine plasma were added. Megakaryocyte aggregation was assessed using phase microscopy by comparing in hemacytometer chambers, the number of single megakaryocytes before and after reaction with ristocetin or bovine plasma.

Enrichment of aggregated megakaryocytes. For enrichment using bovine plasma, megakaryocytes in 700 µL of density-fractionated cell suspension were aggregated with 5% bovine plasma for five minutes. The cuvette was then removed from the aggregometer, placed in a vertical position, and the cells allowed to sediment seven minutes (found to be the optimal time for sedimentation of megakaryocyte aggregates). The supernatant cell suspension was removed and discarded. The sedimented cells were resuspended in 600 µL of CATCH media with 12.5 mmol/L EDTA and 5% bovine plasma and stirred in the aggregometer 30 seconds. The cuvette was removed from the aggregometer and the cell suspension was underlaid with 600 µL of 3% Percoll in CATCH media with 12.5 mmol/L EDTA and 5% bovine plasma. The cells were allowed to sediment seven minutes (found to be the optimal time) after which the supernatant cell suspension was removed. The sedimented cells were resuspended in CATCH media with EDTA and bovine plasma, stirred, underlaid with the 3% Percoll media as above, and allowed to sediment seven minutes. The sedimented cells were resuspended in 5 mL of CATCH media to which 60 µg/mL of dextran sulfate (stock solution = 1 mg/mL in Hanks' BSS without Ca⁺⁺ and Mg⁺⁺) was added. The cell suspension was allowed to stand at least ten minutes. The cells were then concentrated by centrifugation (100 g for five minutes) and resuspended in 0.5 to 1.0 mL of CATCH media for counting, trypan blue exclusion studies, and collection of cells onto slides by cytocentrifugation (Cytospin 2, Shandon Southern Instruments, Inc., Sewickley, Pa). The cytocentrifuge preparations were stained with modified Wright's stain (Hema-Tek stain pak #4481, Miles Scientific, Naperville, Ill) on a Hema-Tek automatic slide stainer, and the percentage of morphologically recognizable megakaryocytes determined to assess megakaryocyte purity.

To compare megakaryocyte enrichment achieved with ristocetin as the aggregating agent to that with bovine plasma, megakaryocytes in CATCH media with 15% guinea pig plasma, 5% fetal calf serum, and 5 mmol/L EDTA were aggregated with 1.75 mg/mL ristocetin for five minutes followed by three differential sedimentations as described above. For the second and third sedimentations, the sedimented cells were resuspended in 600 µL of CATCH media containing 2.0 mg/mL ristocetin, 15% citrated guinea pig plasma, 5% fetal calf serum, and 5 mmol/L Na₂ EDTA. After stirring for 30 seconds, the cells were underlaid with 600 µL of 3% Percoll in CATCH media with the ingredients just described. After the third sedimentation, the megakaryocytes were disaggregated by suspending in 5 mL of CATCH media. The use of 1.75 mg/mL ristocetin during the aggregation step was chosen to give optimal aggregation with minimal plasma protein precipitation. The megakaryocyte

aggregates were better maintained and final megakaryocyte recovery was higher if the ristocetin concentration was slightly higher (2.0 mg/mL) during the second and third sedimentations.

Electron microscopy. Samples were fixed in 2% glutaraldehyde in 0.1 mol/L cacodylate buffer at 4 °C overnight and postfixed with osmium tetroxide for one hour. The samples were dehydrated in ethanol and embedded in resin. Sections were cut and stained 20 minutes in aqueous uranyl acetate, then 20 minutes in Reynolds lead stain.

Measurement of megakaryocyte diameters. For diameter measurements, cells suspended in CATCH media were placed on hemacytometers and visualized by phase-contrast microscopy. Diameters of 75 megakaryocytes in each sample were measured using an eye piece micrometer.

Analysis of DNA content distributions of megakaryocytes by two-color flow cytometry. The DNA content distributions of megakaryocytes in marrow cell suspensions doubly stained with platelet antibody and propidium iodide were analyzed by two-color flow cytometry as previously described.²¹ Briefly, megakaryocytes were specifically labeled with heterologous rabbit antiginea pig platelet antiserum by indirect immunofluorescence using FITC-goat antirabbit IgG/F(ab')₂ (green fluorescence; Tago, Inc., Burlingame, Calif) as the second antibody. One- to two-tenths mL of platelet-depleted guinea pig RBCs were added to the final megakaryocyte suspension after incubation with the platelet antibody to act as a cushion during the washing steps since the purified megakaryocytes were quite fragile. The platelet antiserum was prepared as previously outlined.² After adsorption with guinea pig red cells, the antibody was bound to guinea pig platelets in EDTA, the platelets washed, and the bound antibody eluted with glycine-HCl-saline buffer (pH 2.0) to ensure platelet specificity. The eluted antibody was buffered with phosphate buffer. DNA was stained with propidium iodide (red fluorescence) in hypotonic citrate solution.²² Each stained sample was incubated with RNase (Bovine pancreas, 50 µg/mL, Calbiochem-Behring, San Diego, Calif) for 30 minutes at room temperature prior to flow cytometric analysis to remove RNA-associated propidium iodide staining. The DNA content distribution of megakaryocytes was selectively determined by two-color, green-gated flow cytometric analysis, in which the red and green fluorescence emissions of all cells were analyzed, but only the red fluorescence (DNA content) of cells that specifically bound the platelet antibody was recorded. The green gate for analysis of all samples was selected by examining the green fluorescence distribution of cells with DNA contents ≥8N in unfractionated marrow. The number of cells in each DNA peak was determined by taking the integral from one peak nadir to the next.

RESULTS

Guinea pig megakaryocytes were aggregated by ristocetin and bovine plasma. The aggregates may be several hundred megakaryocytes in size. An example of such a megakaryocyte aggregate is shown in Fig 1. For this preparation, the megakaryocyte-enriched marrow cell fraction suspended in CATCH media was aggregated with 5% bovine plasma.

Characteristics of megakaryocyte aggregation with ristocetin. The dose-response relationship between ristocetin concentration and the degree of megakaryocyte aggregation five minutes after the addition of ristocetin is shown in Fig 2. For this study, the density-fractionated cells were suspended in CATCH medium containing 5% fetal calf serum and 15% guinea pig plasma. Twenty-five percent guinea pig plasma was required to support maximal aggregation with 2.0 mg/mL ristocetin when 5% fetal calf serum was not

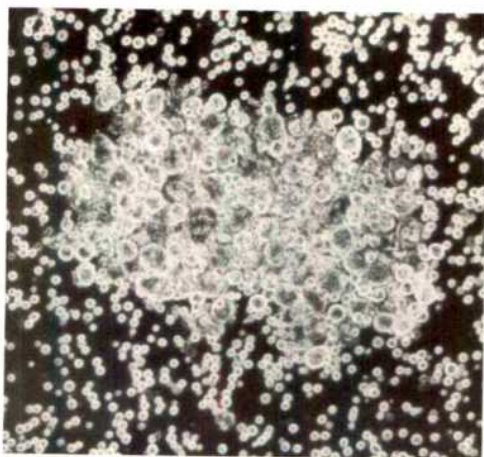


Fig 1. Phase-contrast photomicrograph of megakaryocytes aggregated with 5% bovine plasma (40 $\times$ original magnification; 20 $\times$ current magnification).

included. A rather steep dose response was seen with no megakaryocyte aggregation detectable at 0.5 mg/mL ristocetin. About one half of the megakaryocytes were aggregated with 1.0 mg/mL ristocetin. Maximal aggregation (usually >90% of megakaryocytes aggregated) was seen with 2.0 to 2.5 mg/mL ristocetin. Megakaryocyte aggregation was relatively rapid with more than one half of the megakaryocytes aggregated at 30 seconds and about 3/4 aggregated at one minute. Maximal aggregation occurred at five to seven minutes. The ristocetin-induced megakaryocyte aggregates could be disaggregated by diluting out ristocetin.

Characteristics of megakaryocyte aggregation with bovine plasma. The dose-response relationship between bovine plasma concentration and the degree of megakaryocyte aggregation five minutes after addition of bovine plasma is shown in Fig 3. Maximal aggregation was seen with 2.5% to 5.0% bovine plasma. Some aggregation of other marrow cells occurred with 20% bovine plasma. The time course for megakaryocyte aggregation with 5% bovine plasma was similar to that for ristocetin with maximal aggregation occurring within five to seven minutes. The bovine plasma-aggregated megakaryocytes could be disaggregated by the addition of 60 μ g/mL dextran sulfate.

Megakaryocyte aggregation with purified bovine vWF. Maximal aggregation with purified bovine von Willebrand factor occurred at a concentration of 1 μ g/mL (>90% of megakaryocytes aggregated).

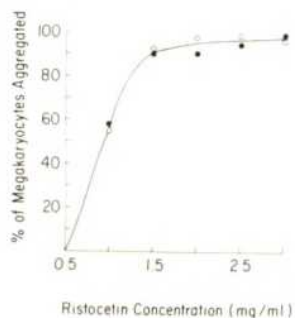
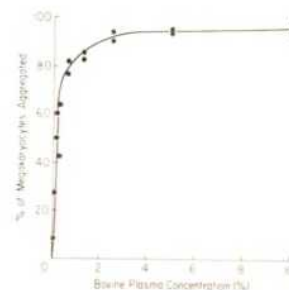


Fig 2. Dose-response relationship between ristocetin concentration and percentage of megakaryocytes aggregated (results of two experiments). Each point represents results from aggregation of 700 μ L of cell suspension (containing 15% guinea pig plasma and 5% fetal calf serum) for five minutes. Megakaryocyte concentration was 183,333 and 231,667/mL.

Fig 3. Dose-response relationship between bovine plasma concentration and percentage of megakaryocytes aggregated (results of two experiments). Each point represents results of aggregation of 700 μ L of cell suspension containing 148,889 to 164,444 megakaryocytes/mL for five minutes.



Megakaryocyte aggregation with normal and von Willebrand disease pig plasma. Plasma of a pig with von Willebrand disease lacked specific megakaryocyte-aggregating activity (Fig 4). Plasma of a normal pig showed a megakaryocyte aggregation dose response like that of bovine plasma. At concentrations of $\geq 10\%$, plasmas of both pigs caused aggregation of other marrow cells.

Enrichment of aggregated megakaryocytes. Several procedures were examined for enrichment of the megakaryocyte aggregates including velocity sedimentation in large volume, shallow gradients, filtration through nylon mesh, and differential sedimentation. Of these, a series of differential sedimentations in the aggregation cuvettes have proved most successful. Two sedimentations of the aggregated cells in a 3% Percoll gradient after an initial sedimentation without a Percoll underlay yielded maximal megakaryocyte enrichment and recovery (Table 1). A third sedimentation in the 3% Percoll gradient resulted in lower megakaryocyte recovery without improvement in megakaryocyte purity.

Higher megakaryocyte enrichment was achieved with bovine plasma as the aggregating agent (Table 2). In other experiments in which bovine plasma and ristocetin were not simultaneously compared, aggregation with bovine plasma usually resulted in higher megakaryocyte recovery (data not shown).

Table 3 summarizes megakaryocyte enrichment achieved in seven recent experiments. Megakaryocytes comprised an average of 0.15% of the cells in unfractionated marrow suspensions. The initial density fractionation step resulted in only a 3-fold enrichment because of the relatively low megakaryocyte recovery. After the aggregation and sedimentation steps, megakaryocytes constituted three fourths of the resulting cell suspensions which represents a 165-fold enrichment from the density fractionation step. Megakaryocyte recovery after the aggregation and sedimentation steps averaged 92% of the input number. Viability of megakaryo-

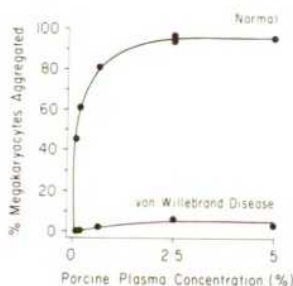


Fig 4. Comparison of megakaryocyte aggregation induced by plasma from a pig with von Willebrand disease to that with normal porcine plasma. Each point represents results of aggregation of 700 μ L of cell suspension containing 127,000 to 167,000 megakaryocytes/mL.

Table 1. Megakaryocyte Purity, Recovery and Viability Achieved by 1, 2, or 3 Sedimentations of Bovine Plasma Aggregated Cells in 3% Percoll

Experiment	Number of Sedimentations in 3% Percoll								
	1			2			3		
	Purity (%) [*]	Recovery (%) [†]	Viability (%) [‡]	Purity (%)	Recovery (%)	Viability (%)	Purity (%)	Recovery (%)	Viability (%)
1	61	84	72	83	81	69	—	—	—
2	56	98	80	85	99	71	—	—	—
3	—	—	—	78	89	81	77	75	65
4	—	—	—	76	114	86	79	69	63

Seven hundred microliters of marrow cell suspension enriched for megakaryocytes by density fractionation were aggregated with 40 μ L of citrated bovine plasma for five minutes in a platelet aggregometer. The cells were allowed to sediment seven minutes and the pellets sedimented 1, 2, or 3 times for seven minutes in 3% Percoll followed by disaggregation as outlined in the Materials and Methods.

^{*}Megakaryocyte purity determined by phase-contrast microscopy on cells in suspension in hemacytometers.

[†]Megakaryocyte recovery based on megakaryocyte concentration of density-fractionated cell suspensions before aggregation with bovine plasma (190,000 to 232,000 megakaryocytes/mL).

[‡]Viability estimated by trypan blue dye exclusion—100 megakaryocytes examined in each cell suspension.

cytes in the final suspension was not significantly different from that in unfractionated marrow.

The appearance of megakaryocytes in a cytocentrifuge preparation made after the complete enrichment procedure is shown in Fig 5. All maturation stages of morphologically recognizable megakaryocytes were represented. The vacuolization observed near the periphery of some megakaryocytes was present before the megakaryocyte aggregation step and

previously has been noted by Levine and Fedorko² after density fractionation and velocity sedimentation and by Berkow et al¹⁰ after counterflow centrifugal elutriation and density fractionation.

Electron microscopic examination of the final megakaryocyte preparations indicated that the plasma and nuclear membranes of most cells were intact; dense granules were evenly distributed in the cytoplasm (Fig 6).

Table 2. Comparison of Megakaryocyte Purity Achieved Following Aggregation With Ristocetin or Bovine Plasma

Experiment	Aggregating Agent	Megakaryocytes Before Aggregation [†]	Recovery* (%)	Final Megakaryocyte Purity (% of Total Cells)	
				Hemacytometer [‡]	Cytospin [§]
1	bovine plasma	196,000	94	80	62
	ristocetin	196,000	75	55	49
2	bovine plasma	188,000	67	79	66
	ristocetin	188,000	93	57	52

^{*}After aggregation for five minutes with 5% bovine plasma or 1.75 mg/mL ristocetin, megakaryocytes were enriched by three differential sedimentations followed by disaggregation as outlined in the Materials and Methods. Recovery represents the number of megakaryocytes present in the final suspensions after the sedimentation steps expressed as a percentage of the megakaryocytes in the density-fractionated marrow before aggregation.

[†]Number of megakaryocytes in the 700 μ L of density-fractionated marrow suspension that was aggregated with each aggregating agent.

[‡]Megakaryocyte purity as determined by phase-contrast microscopy of cells in suspension on a hemacytometer.

[§]Megakaryocyte purity as determined from Wright-stained cytocentrifuge preparation of the final cell suspensions.

Table 3. Summary of Megakaryocyte Enrichment

Experiment	Unfractionated Marrow					Megakaryocyte Enrichment by Density Fractionation				Final Megakaryocyte Suspensions After MK Aggregation and Sedimentations			
	Total Cell $\times 10^6$ [*]	Viability of Other Marrow Cells (%)	Total MK $\times 10^3$	% MK	MK Viability (%)	Total $\times 10^6$	Number of MK $\times 10^3$	% MK	% MK Recovery From Previous Step	% MK		% MK Recovery From Previous Step	MK Viability (%)
										Hemacytometer	Cytospin		
1	355	85	933	0.26	60	67	428	0.64	46	80	79	70	62
2	550	91	400	0.07	54	97	210	0.22	53	69	78	109	60
3	505	92	900	0.17	74	75	368	0.49	41	76	74	114	86
4	437	94	567	0.13	86	66	348	0.53	61	85	83	99	71
5	480	92	467	0.10	66	66	242	0.37	52	77	76	68	73
6	516	92	1000	0.19	74	64	395	0.62	39	78	69	89	81
7	375	90	600	0.16	56	68	222	0.33	37	—	77	92	68
Mean	460	91	695	0.15	67	72	316	0.46	47	78	76	92	72
1 SD	74	3	243	0.06	12	12	89	0.16	9	5	4	18	9

Guinea pigs weighed 300 to 338 g.

^{*}Total cells obtained from femora, tibiae, and humeri of one guinea pig.



Fig 5. Photomicrograph of a Wright's stained cytocentrifuge preparation of a final megakaryocyte suspension (after density fractionation and the aggregation enrichment procedure; original magnification 250 $\times$; current magnification 112 $\times$).

Next, sizes of the megakaryocytes before and after the aggregation enrichment procedure were compared. Diameters of megakaryocytes in the cell suspension resulting from the density fractionation step were compared to those of megakaryocytes in the final cell suspension (after aggregation, three sedimentations, and disaggregation; Table 4). Both mean diameter and diameter distribution of megakaryocytes in the final preparation were similar to those before aggregation.

Also, we have compared by flow cytometry the DNA content distributions of megakaryocytes enriched by aggregation with bovine plasma to those of megakaryocytes in unfractionated marrow, and after the equilibrium density centrifugation step. It should be pointed out that because we were primarily interested in the DNA distribution of polyploid cells, we chose flow cytometer settings which optimally detected polyploid cells even though this results in some 2N and 4N cells with nonspecific immunoglobulin binding being included as platelet antibody-positive cells. Thus, for the purposes of these analyses, only cells with DNA contents $\geq 8N$ are considered. The ratio of 8N:16N:32N cells after the megakaryocyte enrichment steps was similar to that of unfractionated marrow (Table 5).

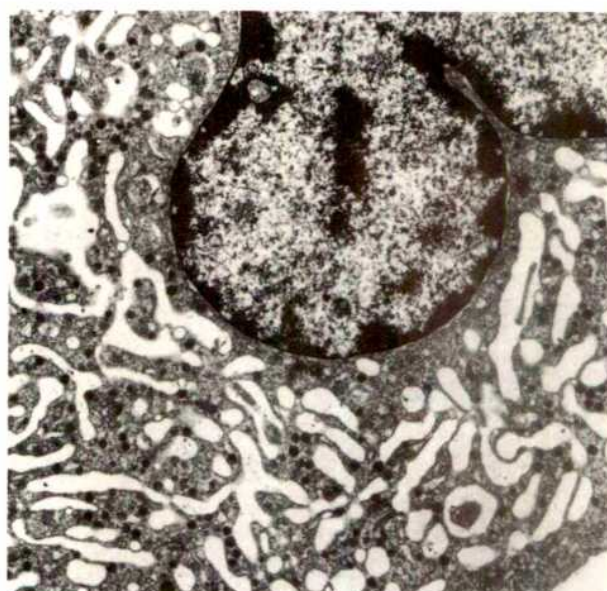


Fig 6. Ultrastructure of a relatively mature megakaryocyte after enrichment by density fractionation and the aggregation enrichment procedure (Original magnification 8300 $\times$; current magnification 4980 $\times$).

DISCUSSION

In this study we have asked whether guinea pig megakaryocytes can be aggregated by ristocetin and bovine plasma and if so, can such aggregation be used as a step for megakaryocyte enrichment and purification. Almost all megakaryocytes could be aggregated with ristocetin at a concentration of 2.0 mg/mL in 25% guinea pig plasma or with 5% bovine or porcine plasma. The component in bovine and porcine plasma that induces megakaryocyte aggregation, as with platelet aggregation, appears to be vWF since purified bovine vWF aggregated megakaryocytes while plasma from a pig with von Willebrand disease did not. The aggregated megakaryocytes could be disaggregated by diluting out ristocetin or adding dextran sulfate to bovine plasma-aggregated samples.

Although the mechanism is not well understood, platelet surface glycoprotein Ib seems to be the primary binding site

Table 4. Average Diameter and Diameter Distribution of Megakaryocytes Before and After Aggregation With Bovine Plasma

	Average Diameter (μm)*	Diameter Distribution (% of Total)*					
		16 to 20 μm	21 to 25 μm	26 to 30 μm	31 to 35 μm	36 to 40 μm	41 to 45 μm
Before							
aggregation†	25.0 $\pm$ 1.0	38.7 $\pm$ 4.8	24.8 $\pm$ 4.3	18.1 $\pm$ 4.0	11.2 $\pm$ 2.0	6.7 $\pm$ 3.4	0.5 $\pm$ 0.7
After							
aggregation, 3 sedimentations and disaggregation‡	25.8 $\pm$ 0.4	36.0 $\pm$ 1.9	20.8 $\pm$ 7.0	15.2 $\pm$ 2.0	15.5 $\pm$ 4.0	12.0 $\pm$ 3.6	0.8 $\pm$ 1.2

*Mean $\pm$ 1 SD for five experiments.

†Megakaryocytes were enriched by density fractionation, pelleted, resuspended in CATCH media with 12.5 mmol/L EDTA, pelleted, and resuspended in CATCH media.

‡Megakaryocytes were enriched by density fractionation, pelleted, resuspended in CATCH media with 12.5 mmol/L EDTA followed by aggregation with 7.5% bovine plasma, 3 sedimentations, disaggregation with 60 $\mu\text{g}/\text{mL}$ dextran sulfate, pelleted, and resuspended in CATCH media.

Table 5. Comparison of DNA Content Distribution of Platelet Antibody Positive Cells With DNA Content $\geq$ 8N at Various Points in the Purification Procedure

Marrow Suspension	No. of Cells	8N (%)	16N (%)	32N (%)
Experiment 1				
Unfractionated marrow	3669*	35.2	58.9	5.9
After density fractionation	5654	30.6	64.1	5.2
After aggregation and sedimentations	4517	31.3	62.7	6.0
Experiment 2				
Unfractionated marrow	1074	34.5	55.0	10.4
After density fractionation	6143	30.4	57.1	12.5
After aggregation and sedimentations	1939	28.0	60.8	11.2

*Number of platelet antibody-positive cells with DNA content $\geq$ 8N.

for von Willebrand factor during ristocetin-induced aggregation.²³⁻²⁷ Bovine vWF also probably reacts with platelet glycoprotein Ib since platelets from patients with Bernard-Soulier syndrome that are deficient in this glycoprotein are not aggregated by this factor.^{28,29} Glycoprotein Ib has been detected on megakaryocytes^{5,30,31} and thus is most likely the site with which ristocetin and bovine plasma interact to produce aggregation of megakaryocytes. It should be pointed out that heparin inhibits platelet aggregation with ristocetin³² and bovine vWF¹⁶ and therefore should not be used as the anticoagulant for marrow collection.

Megakaryocytes enriched using the bovine plasma aggregation step have diameters and DNA content distributions similar to those in unaggregated marrow suspensions. This suggests that megakaryocyte populations produced by this procedure are representative of the original populations. Although we have made no direct comparisons of DNA content distributions of megakaryocyte populations obtained using velocity sedimentation after density fractionation with those enriched by the procedure described here, results of others^{7,12,33} suggest that loss of lower ploidy megakaryocytes can be substantial with the velocity sedimentation step. Taken together, these results suggest that bovine plasma aggregation of megakaryocytes before the sedimentation step allows equal recovery of megakaryocytes of various DNA contents and therefore provides an advantage over velocity sedimentation of single megakaryocytes.

The ability to induce large aggregates of megakaryocytes with ristocetin or bovine plasma also should be useful in electron microscopic studies of these cells since one of the

major obstacles to such studies currently is locating a cell type of such low frequency once the processed marrow specimens are ready for examination.

In situations where megakaryocyte number is reduced to such an extent that megakaryocyte aggregates will not form or are small, the extent of aggregation and size of aggregates might be increased by adding platelets before aggregation so that megakaryocyte-platelet aggregates would be formed. After separation of such aggregates from other cells, the megakaryocytes and platelets could be disaggregated and platelets separated from megakaryocytes if desired.

The ability of ristocetin and bovine vWF to aggregate platelets in plasma is limited to a few species³⁴ possibly because of interference by plasma proteins.^{35,36} Fortunately, platelets of man and guinea pig are readily aggregated by these reagents, but those of other small laboratory animals such as the rat¹⁹ are not. We expect that aggregation of megakaryocytes by these reagents will show the same species restrictions. Indeed, in our hands, rat megakaryocytes were not aggregated by either ristocetin or bovine plasma. However, botrocetin (venom coagglutinin),^{37,38} a snake venom component from certain *Bothrops* species which, in the presence of vWF, aggregates platelets of all mammalian species examined,³⁹ may aggregate megakaryocytes of other species.

Bovine plasma has several advantages over ristocetin as an agent to aggregate megakaryocytes for subsequent enrichment. First, the megakaryocyte purity achieved was higher with bovine plasma. The lower megakaryocyte purity with ristocetin appeared to result from the formation of some small aggregates of other marrow cells that either adhered to megakaryocytes or sedimented with them. Such aggregates were less frequently observed in megakaryocyte preparations aggregated with bovine plasma. The presence of these other marrow cell aggregates following ristocetin aggregation may be related to the precipitation of plasma proteins by this antibiotic.¹⁷ Second, the use of bovine plasma does not require the addition of another source of vWF, so the system is less complicated. Third, bovine plasma is much less expensive than ristocetin.

In conclusion, megakaryocytes are aggregated by ristocetin and bovine plasma as are platelets. We have used this observation to develop an alternative approach to megakaryocyte enrichment that results in megakaryocyte populations representative of those in unfractionated marrow.

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Inhibition of Thromboxane Formation In Vivo and Ex Vivo: Implications for Therapy With Platelet Inhibitory Drugs

By Irene A.G. Reilly and Garret A. FitzGerald

The capacity of platelets to generate thromboxane A_2 , reflected by measurement of serum thromboxane B_2 (TxB_2), greatly exceeds the systemic production of thromboxane in vivo. Thus, it is possible that substantial but incomplete inhibition of thromboxane formation ex vivo would still allow marked augmentation of thromboxane production in vivo. To address this hypothesis, we administered aspirin 120 mg, a selective inhibitor of thromboxane synthase (TxSI), 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid (UK-38,485) 200 mg, and a combination of both drugs to 12 healthy volunteers and measured the effects on serum TxB_2 and urinary 2,3-dinor-thromboxane B_2 (Tx-M), an index of endogenous thromboxane biosynthesis. Although serum TxB_2 was maximally inhibited by $94 \pm 1\%$ after aspirin and $96 \pm 2\%$ after the TxSI, maximal depression of Tx-M was only $28 \pm 8\%$ and $37 \pm 9\%$, respectively. Combination of aspirin with the TxSI resulted in a small but significant increase in inhibition of thromboxane generation ex vivo ($98 \pm 1\%$ v

$94 \pm 1\%$; $P < 0.05$), but a disproportionately greater fall in thromboxane synthesis in vivo ($58 \pm 7\%$; $P < 0.01$). Consistent with further inhibition of platelet thromboxane synthesis, addition of the TxSI abolished the transient decline in prostacyclin formation after aspirin alone. Administration of a lower dose of aspirin (20 mg) to 6 healthy subjects caused a small reduction in Tx-M ($12 \pm 4\%$; $P < 0.05$) and inhibited serum TxB_2 by $48 \pm 2\%$. The relationship between inhibition of platelet capacity to form thromboxane ex vivo (serum TxB_2) and synthesis in vivo (Tx-M) departed markedly from the line of identity. When total blockade of the capacity of platelets to generate thromboxane is approached, minor decrements in capacity result in a disproportionate depression of actual thromboxane biosynthesis. These results imply that pharmacologic inhibition of serum TxB_2 must be virtually complete before thromboxane-dependent platelet activation is influenced in vivo.

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THE capacity of tissues to generate eicosanoids in response to physical and chemical stimuli is known to greatly exceed the actual rates of biosynthesis observed in vivo.¹ Formation by human platelets of the potent eicosanoid, thromboxane A_2 , measured as its stable metabolite thromboxane B_2 , is a good example of this phenomenon. Under physiological conditions, the maximal endogenous concentration of thromboxane B_2 in plasma has been estimated to be in the order of 1 to 2 pg/mL.² However, the levels of thromboxane B_2 measured in serum, which reflect the capacity of platelets to form thromboxane in response to thrombin, are over a thousand-fold higher at 300 to 400 ng/mL.³ Thus, the normal rates of thromboxane synthesis observed in vivo can be accounted for by activation of platelets to less than 0.1% of their capacity for thromboxane generation.

The distinction between the capacity for thromboxane production ex vivo and endogenous thromboxane biosynthesis is an important one. In several human syndromes of platelet activation the capacity of platelets to form thromboxane is unchanged, but thromboxane biosynthesis in vivo is increased.^{4,5} Earlier work from our laboratory has suggested that the relationship between the inhibition of the capacity of platelets to generate thromboxane and the persistence of thromboxane-dependent platelet activation may not be linear. An increase in the degree of inhibition of serum thromboxane formation from a mean of 95% to 99% inhibi-

tion, for example, results in a further significant increase in the extent of inhibition of platelet aggregation and a further prolongation of the bleeding time.⁶ In addition, others have shown that even a residual 10% capacity to generate thromboxane is enough to fully sustain thromboxane-dependent platelet aggregation.⁷ These observations have important implications for the use of antiplatelet drugs and inhibitors of the cyclooxygenase and thromboxane synthase enzymes in human syndromes of platelet activation. Many of the clinical and pharmacological studies of platelet inhibitory therapy in such conditions have employed measurement of serum thromboxane B_2 as a guide to the adequacy of dosing.^{8,9} A discrepancy between inhibition of serum thromboxane B_2 and platelet thromboxane production in vivo raises the possibility that despite substantial, but incomplete inhibition of the capacity of platelets for thromboxane generation, actual thromboxane biosynthesis may remain significantly enhanced.

The aim of the present study was to investigate the relationship between inhibition of serum thromboxane generation ex vivo and endogenous thromboxane biosynthesis in order to explore the hypothesis that incomplete inhibition of the capacity of platelets to generate thromboxane allows significant synthesis to continue in vivo.

MATERIALS AND METHODS

Study Design

Twelve healthy male volunteers (aged 21 to 46 years, weight 80.5 ± 5 kg) participated in the study which was approved by the Committee for the Protection of Human Subjects of Vanderbilt University. The main study, which was double blind, randomized, and placebo controlled, was performed on four study days, each separated by a washout period of ten days. On each study day, the volunteers received as a single dose of one of the following: (a) aspirin 120 mg; (b) a thromboxane synthase inhibitor, 3-(1H-imidazole-1-yl-methyl)-2-methyl-1H-indole-propanoic acid¹⁰ (UK-38,485) 200 mg; (c) aspirin 120 mg plus UK-38,485 200 mg, or (d) placebo, each administered as 5 identical unlabelled white capsules

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(kindly provided by Dr P. Urquilla of Pfizer Inc., Groton, Conn). All the subjects had fasted for a minimum of ten hours prior to dosing and for four hours after dosing and none had taken aspirin or aspirin-like drugs in the 2-week period prior to or during the study.

Blood was drawn before and 1, 4, 6, 8, and 24 hours after dosing by fresh venipuncture from an antecubital vein for plasma drug levels and serum thromboxane determinations. Urine was collected for measurement of prostacyclin and thromboxane metabolites for the 24 hours before each study day and in 3 postdosing aliquots: 0 to 6, 6 to 12, and 12 to 24 hours. Physical examination, laboratory evaluation, and an EKG were carried out prior to inclusion in the study. Safety tests (full blood count, routine automated biochemistry screen, and urinalysis) were performed before dosing and on completion of each study day. Blood pressure, measured by mercury sphygmomanometer in the sitting position, and heart rate were monitored at each blood sampling time point prior to venipuncture.

On a separate occasion, aspirin was administered to 6 volunteers such as to result in partial inhibition of platelet thromboxane formation *ex vivo*. Four 5 mg aspirin capsules (prepared by the Vanderbilt Pharmacy) were administered two hours apart. Blood was drawn for estimation of serum thromboxane B_2 formation before dosing and eight hours after the first dosage. Urine was collected for eicosanoid analysis for 24 hours prior to dosing and from 0 to 12 and 12 to 24 hours after initiation of dosing.

Finally, blood and urine samples were obtained for measurement of serum thromboxane B_2 and urinary 2,3-dinor-thromboxane B_2 , respectively, from 28 additional apparently healthy volunteers (14 males, 14 females; age 24 to 38 years; wt. 45 to 96 kg) and 4 patients attending the hematology outpatient clinic, two with secondary thrombocytosis, one with hypersplenism, and one with idiopathic thrombocytopenic purpura.

Biochemical Analyses

2,3-dinor-thromboxane B_2 (Tx-M). Tx-M was measured employing a stable isotope dilution assay described in detail elsewhere.¹¹ Briefly, 12.5 ng of a deuterated internal standard was added to a 5 mL aliquot of urine that was then converted to the methoxime derivative by the addition of methoxyamine hydrochloride in acetate buffer. The sample was then applied to a phenylboronic acid column, washed with acid salt followed by methanol, and eluted with 0.1 N sodium hydroxide in methanol. The eluate was acidified, applied to a silica Sep-Pak, washed, and eluted with ethyl acetate. The sample was purified by thin layer chromatography, derivatized to the methoxime, pentafluorobenzyl ester, and further purified by repeat thin-layer chromatography. Derivatization was completed by formation of the trimethylsilyl ether and quantitation accomplished on the Nermag R10-10C operating in the negative ion mode, monitoring m/z 586 for endogenous Tx-M and 590 for the deuterated internal standard.

2,3-dinor-6-keto-PGF_{1 α} (PGI-M). PGI-M was also measured by stable isotope dilution assay employing negative ion-chemical ionization gas chromatography-mass spectrometry as described elsewhere.¹² Briefly, a 5 mL aliquot of urine was first spiked with 5 ng of a deuterated internal standard. The sample was then subjected to extraction and back extraction under alkaline and acidic conditions, and derivatized as the methoxime pentafluorobenzyl ester. Further purification was carried out by thin-layer chromatography and the derivatization completed by formation of the trimethylsilyl ether derivative. Quantitation was accomplished by stable isotope dilution using a Hewlett Packard (Avondale, PA) 5980 instrument operating in the negative ion mode, monitoring m/z 586 for endogenous PGI-M and m/z 590 for the deuterated internal standard.

Serum thromboxane B_2 . Serum thromboxane B_2 generation *ex vivo* was measured as previously described.³ Venous blood was collected into a warmed glass tube, incubated at 37 °C for one hour,

and then spun at 2,000 g for 15 minutes. Serum was separated and stored at -20 °C for later analysis by radioimmunoassay.

Plasma Drug Levels

Plasma concentrations of UK-38,485 were analyzed by high-performance liquid chromatography (HPLC) using UK-37,418 as an internal standard. The drug was isolated from plasma by precipitation with acetonitrile, centrifuged at 2,000 g for five minutes, and evaporated to dryness under nitrogen. After being resuspended in tetramethylethylenediamine (TEMED)/citrate buffer, aliquots were injected onto a Spherisorb Phenyl column (Hichrom, Reading, UK) with a mobile phase of 75% methanol: 25% TEMED/citrate buffer 0.1 mol/L. UK-38,485 and the internal standard were detected by fluorescence at an excitation wavelength of 220 nm and emission of 370 nm. These analyses were kindly undertaken by Dr P. Gibson (Chapel Laboratories, Hythe, Kent, UK) by arrangement with Dr J.F. Faulkner, Pfizer Central Research, Groton, CT. Plasma concentrations of salicylate were measured by a stable isotope dilution assay employing gas chromatography mass spectrometry.¹³

Statistical Analysis

Data were analyzed by nonparametric methods¹⁴ thereby avoiding assumptions as to the distribution of the variables involved. Two-way analysis of variance was by the method of Friedman and subsequent pair-wise analysis was by the Wilcoxon Rank Sum Test. Unless otherwise stated, our results are expressed as the mean $\pm$ the standard error of the mean (SEM).

RESULTS

Platelet Thromboxane Formation

Platelet thromboxane generation *ex vivo* (measured as serum thromboxane B_2) was reduced by $94 \pm 1\%$ one hour after dosing with 120 mg aspirin from 349 ± 37 ng/mL to 18 ± 4 ng/mL. Maximal depression of serum thromboxane B_2 after the thromboxane synthase inhibitor (200 mg UK-38,485) was not significantly different, falling by $96 \pm 2\%$ from 312 ± 33 ng/mL to 12 ± 3 ng/mL one hour after drug administration. While the inhibition induced by aspirin persisted throughout the study period, serum thromboxane B_2 had returned to a mean of 70% of predosing values 24 hours after administration of the thromboxane synthase inhibitor (Fig 1). Combination of aspirin with the synthase inhibitor achieved a minor but significant increase in the degree of inhibition *ex vivo* over that observed with aspirin administration alone: serum thromboxane B_2 fell by $98 \pm 1\%$ from 380 ± 19 to 8 ± 4 ng/mL one hour after dosing. The difference between the combination and aspirin alone, although small, remained statistically significant ($P < 0.05$) one, four, and six hours after drug administration (Fig 1). Serum thromboxane B_2 formation did not change significantly following placebo and there was no significant difference between baseline values on each of the four study days. Administration of the lower dose of aspirin (4×5 mg) resulted in a concomitantly smaller reduction in serum thromboxane B_2 by $48 \pm 2\%$ from 365 ± 21 to 190 ± 8 ng/mL.

Thromboxane Synthesis In Vivo

Peak depression of Tx-M excretion following aspirin 120 mg, which was observed in the six- to 12-hour aliquot after

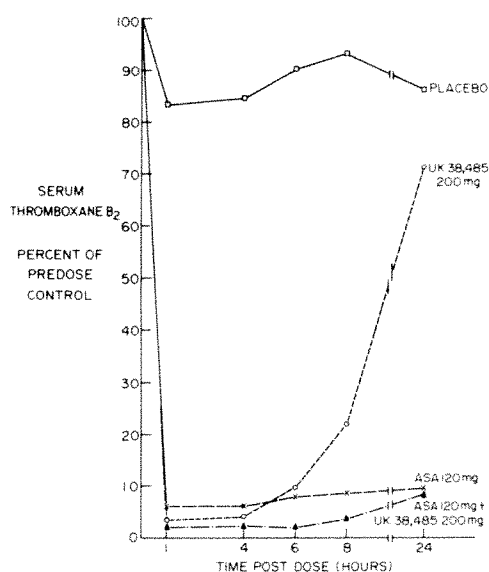


Fig 1. The effect on platelet cyclooxygenase formation (serum thromboxane B_2) of single dose administration of placebo, UK-38,485 (200 mg), low-dose aspirin (ASA 120 mg), and a combination of low-dose aspirin with UK-38,485 (200 mg) in 12 healthy male subjects. Serum thromboxane is expressed as a percentage of the predosing control value for each treatment regimen. There was no significant difference in serum thromboxane B_2 from control values following placebo administration. Inhibition of serum thromboxane was significantly greater after the aspirin/UK-38,485 combination compared to aspirin alone ($P < 0.05$; one, four, and six hours after dosing) and compared to UK-38,485 ($P < 0.01$; four, six, eight, and 24 hours after dosing). The inhibitory effect of aspirin 120 mg v UK-38,485 (200 mg) on serum thromboxane B_2 was significant only eight and 24 hours after administration ($P < 0.01$).

dosing, was only $28 \pm 8\%$ from predosing values despite almost maximal inhibition of platelet cyclooxygenase throughout the study period (Table 1). The maximal depression of Tx-M excretion after the thromboxane synthase inhibitor was somewhat greater at $37 \pm 9\%$ ($P = 0.06$). Despite the minor increase in inhibition of the capacity of platelets to form thromboxane ex vivo ($94 \pm 1\%$ v $98 \pm 1\%$, $P < 0.05$), addition of the thromboxane synthase inhibitor to the cyclooxygenase inhibitor resulted in a disproportionate fall in thromboxane metabolite excretion ($58 \pm 7\%$) com-

Table 1. Excretion of 2,3-Dinor-Thromboxane B_2 (Tx-M) in 12 Healthy Male Subjects After Single-Dose Administration of Aspirin 120 mg, UK-38,485 200 mg, and Aspirin 120 mg in Combination With UK-38,485 200 mg

Time After Dosing (hr)	Aspirin 120 mg	UK-38,485 200 mg	UK-38,485 200 mg + Aspirin 120 mg
-24	134 ± 21	97 ± 14	95 ± 12
0-6	$108 \pm 18^{\dagger}$	85 ± 10	$65 \pm 9^*$
6-12	$92 \pm 17^{\dagger}$	$56 \pm 6^*$	$47 \pm 11^*$
12-24	$104 \pm 17^{\dagger}$	76 ± 9	$37 \pm 6^*$

Values are expressed in pg/mg creatinine (mean $\pm$ SEM). There were no significant differences between the predosing values.

* $P < 0.01$ compared to control values.

$\dagger P < 0.05$ aspirin 120 mg v aspirin 120 mg + UK-38,485 200 mg.

$\ddagger P < 0.01$ aspirin 120 mg v aspirin 120 mg + UK-38,485 200 mg.

pared to that after aspirin alone ($P < 0.01$). To address the possibility that alterations in Tx-M excretion after aspirin resulted from altered renal tubular handling of the metabolite rather than inhibition of cyclooxygenase¹⁵ we administered an identical (120 mg) dose of sodium salicylate, which is a weak inhibitor of platelet cyclooxygenase compared with aspirin but is subject to the same renal tubular excretory mechanism. In contrast to aspirin 120 mg, sodium salicylate 120 mg did not alter Tx-M excretion or the capacity of platelets to form thromboxane B_2 (Table 2).

Tx-M excretion did not differ significantly prior to drug administration on each of the study days ($P > 0.2$; Table 1). Although there was moderate intersubject variability, the ranges of Tx-M excretion on each of the study days (respectively, 73 to 241, 44 to 167, and 55 to 174 pg/mg creatinine after aspirin 120 mg alone, 200 mg UK-38,485 and the combination of aspirin with UK-38,485) was comparable to that of previous studies by ourselves and others.^{10,11,16} The intrasubject variability was small, with the exception of the two subjects who smoked, a factor that we have subsequently identified as being associated with increased Tx-M excretion.¹⁷ Both of these subjects had higher levels of Tx-M excretion (353 and 363 pg/mg creatinine) during the second (5 mg $\times$ 4 aspirin) study in which smoking had not been restricted. However, the maximum degree of inhibition after aspirin administration was similar whether these subjects were included in the analysis or not: 218 ± 36 falling to 187 ± 52 12 to 24 hours after dosing ($12 \pm 4\%$) v 134 ± 21 falling to 112 ± 22 pg/mg creatinine ($14 \pm 4\%$).

Tx-M excretion was found to be proportional to platelet count over a wide range of platelet counts in apparently healthy subjects (Fig 2). In addition, in two patients with thrombocytopenia due to peripheral platelet destruction and a shortened platelet life span (one with ITP and the other with hypersplenism), Tx-M excretion was correspondingly elevated at 545 and 1600 pg/mg creatinine, respectively (Fig 2). Metabolite excretion was markedly increased in two patients with secondary thrombocytosis. The pattern of recovery of metabolite excretion after reversible (UK-38,485) and irreversible inhibition of platelet cyclooxygenase (Table 1) was also consistent with urinary 2,3-dinor-Tx B_2 principally deriving from platelets.

Table 2. Urinary Excretion of 2,3-Dinor-Thromboxane B_2 (Tx-M), Serum Thromboxane (Tx) B_2 , and Plasma Concentrations of Salicylate in 3 Healthy Male Subjects After Single-Dose Administration of Salicylate 120 mg

	Prior to Dosing	After Dosing	
		Time (hr)	Concentration
Plasma Salicylate (μ g/mL)	ND	3	7.6 ± 1.2
Serum Tx B_2 (ng/mL)	345 ± 75	3	313 ± 29
Tx-M (pg/mg creatinine)	75 ± 13	0-6	85 ± 28
		6-12	78 ± 10
		12-24	72 ± 7

Values are expressed as mean $\pm$ SEM. ND, not detectable; assay sensitivity 10 pg/mL.¹³

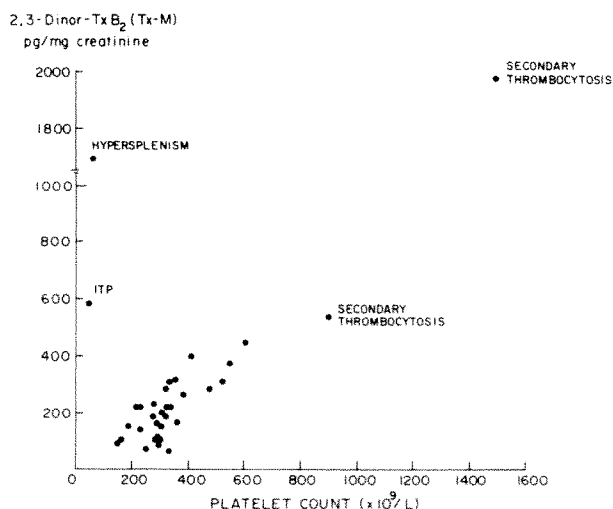


Fig 2. The relationship between platelet count and urinary excretion of 2,3-dinor-Tx_B₂ in 32 subjects. Metabolite excretion was discordant with the platelet count in two patients with syndromes associated with an increase in platelet turnover (hypersplenism and idiopathic thrombocytopenic purpura: ITP). When these patients were excluded, the 2,3-dinor-Tx_B₂ excretion significantly correlated ($r = .91$; $P < 0.001$) with platelet count.

Prostacyclin Biosynthesis

Aspirin 120 mg caused a small transient ($25 \pm 11\%$) reduction in PGI-M excretion that was significant in the zero- to 6-hour urine aliquot after dosing ($P < 0.01$). This effect was not apparent when aspirin was administered in combination with the thromboxane synthase inhibitor (Table 3). PGI-M excretion was also unaltered by the lower dose of aspirin (4×5 mg; 120 ± 20 v 128 ± 20 pg/mg creatinine) but was significantly increased ($P < 0.05$) following administration of the thromboxane synthase inhibitor from 119 ± 18 to 143 ± 19 pg/mg creatinine six to 12 hours after dosing (Table 3). There was no significant difference in PGI-M excretion between either the control days that preceded each dosing period or in the three timed aliquots following placebo administration.

Comparison of Inhibition of Thromboxane Formation Ex Vivo and In Vivo

Mean data relating peak inhibition of thromboxane generation ex vivo to peak inhibition of metabolite excretion are

Table 3. Excretion of 2,3-Dinor-6-Keto-PGF_{1 α} (PGI-M) in 12 Healthy Male Subjects After Single-Dose Administration of UK-38,485 (200 mg), Aspirin 120 mg, or UK-38,485 Plus Aspirin 120 mg

Time After Dosing (hr)	Aspirin 120 mg	UK-38,485 200 mg	UK-38,485 200 mg + Aspirin 120 mg
-24	100 $\pm$ 10	119 $\pm$ 18	116 $\pm$ 20
0-6	70 $\pm$ 11†	122 $\pm$ 16	92 $\pm$ 14
6-12	104 $\pm$ 22	143 $\pm$ 19*	111 $\pm$ 23
12-24	110 $\pm$ 16	131 $\pm$ 19	108 $\pm$ 14

Values are expressed in pg/mg creatinine (mean $\pm$ SEM).

* $P < 0.05$ compared to placebo.

† $P < 0.01$ compared to placebo.

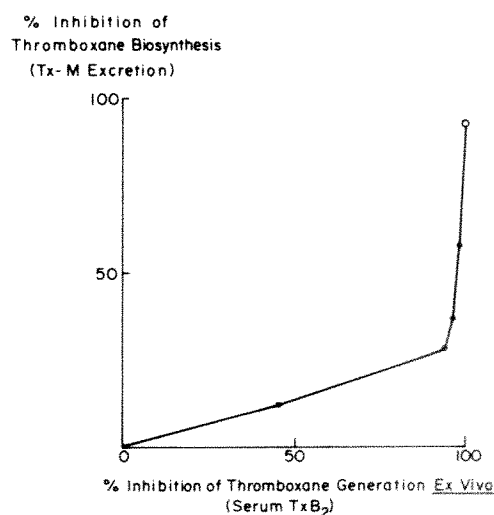


Fig 3. The relationship between inhibition of platelet capacity to generate thromboxane (serum thromboxane B₂) ex vivo and thromboxane synthesis in vivo (2,3-dinor-thromboxane (Tx-M) excretion). Data were derived from experiments involving two doses of aspirin (see text), 200 mg of UK-38,485, a thromboxane synthase inhibitor, and a combination of aspirin with the thromboxane synthase inhibitor. Data indicating complete inhibition (0) of both serum thromboxane B₂ (99%) and dinor thromboxane excretion (97%) were obtained after chronic administration of aspirin 2,600 mg/d.⁶

illustrated in Fig 3. Maximum depression of metabolite excretion was measured in the aliquot collected six to 12 hours after dosing with aspirin 120 mg or with UK 38,485, but in the 12- to 24-hour aliquot with the other dosage regimens. The delay in depression of metabolite excretion is likely to reflect a delay both in transformation (as shown thromboxane B₂ infusion²) and in renal clearance.

The relationship between the inhibition of platelet capacity to form thromboxane ex vivo and actual biosynthesis departed markedly from the line of identity. As maximal blockade of the capacity to generate thromboxane ex vivo was approached, minor increments in the degree of inhibition achieved resulted in a disproportionately greater depression of thromboxane synthesis in vivo.

Plasma Drug Levels

Plasma drug levels of UK-38,485 are shown in Table 4. There was no significant difference in the drug concentrations measured whether UK-38,485 was administered either as a single dose or together with 120 mg of aspirin, confirming the absence of any pharmacokinetic interaction between

Table 4. Plasma Concentrations of UK-38,485 (ng/ml)

Treatment Group	Time After Dosing (hr)			
	1	4	6	8
UK-38,485 200 mg	3207 $\pm$ 333	225 $\pm$ 46	66 $\pm$ 8	26 $\pm$ 4
UK-38,485 200 mg plus aspirin 120 mg	2992 $\pm$ 320	199 $\pm$ 22	66 $\pm$ 7	28 $\pm$ 3

Values are expressed as mean $\pm$ SEM in 12 healthy male subjects. Limit of sensitivity 10 ng/mL.

these drugs. Peak drug levels were measured one hour after dosing and the decline in plasma levels following administration of UK-38,485 was rapid, less than 3% of the peak plasma drug levels being detectable after six hours. The temporal discrepancy between the offset in plasma drug levels and the inhibition of serum thromboxane B_2 generation has previously been observed with thromboxane synthase inhibitors and appears to result from supramaximal concentrations of the parent compound at the time of peak drug levels, rather than the presence of active metabolites.¹⁸

DISCUSSION

We have previously established that there is a marked discrepancy between the capacity of platelets to generate thromboxane B_2 and the actual production rate of thromboxane *in vivo*² based on excretion of its urinary metabolite, 2,3-dinor-thromboxane B_2 . If platelets are the major source of this metabolite in urine, this observation suggests that activation of platelets *ex vivo* during blood sampling and processing, rather than endogenous biosynthesis, largely accounts for the plasma levels of thromboxane B_2 widely reported in the literature. The calculated physiological secretion rate of thromboxane B_2 is extremely low, approximately 0.1 ng/kg/min, which would result in a maximal plasma concentration of 1 to 2 pg/mL.² Thus, in healthy individuals in whom minimal evidence of platelet activation *in vivo* would be expected, activation of platelets to less than 0.1% of their capacity would be sufficient to account for these plasma levels.

This concept has implications for therapy with platelet inhibitory drugs in conditions in which platelet activation is of importance. In several human syndromes in which platelet activation *in vivo* has been demonstrated and is thought to play a primary role, endogenous biosynthesis of thromboxane appears to be markedly enhanced.^{4,5,19} For example, urinary 2,3-dinor-TxB₂ excretion is increased on average 4- to 5-fold during episodes of myocardial ischemia in patients with unstable angina.¹⁹ Such increments are of likely pathophysiologic importance in this disease as cardiovascular mortality is significantly reduced by aspirin.²⁰ However, even in the presence of a 95% inhibition of serum thromboxane B_2 , thromboxane biosynthesis could, in theory, be increased 50-fold above basal levels in response to stimuli to its production *in vivo*. Thus, pathophysiologically important increments in thromboxane formation might still occur in patients receiving sufficient aspirin to cause substantial but incomplete inhibition of serum thromboxane B_2 *ex vivo*.

To explore this possibility more directly, we designed the present study to examine the relationship between inhibition of platelet capacity to form thromboxane (serum thromboxane B_2) and inhibition of thromboxane synthesis *in vivo*, as reflected by excretion of its urinary metabolite, 2,3-dinor-thromboxane B_2 . We postulated that as maximal blockade of the capacity to generate thromboxane *ex vivo* was approached, a disproportionately greater inhibition of actual thromboxane synthesis by platelets *in vivo* would be expected. On the basis of previously published work,^{6,21} we selected two doses of aspirin for these studies: the lower dose (5 mg $\times$ 4) was chosen to produce a minor but significant

reduction in *ex vivo* thromboxane formation; and the higher dose (120 mg) as one predicted to cause substantial but incomplete inhibition of thromboxane generation *ex vivo*. In order to inhibit thromboxane formation *ex vivo* still further, we coadministered with aspirin an inhibitor acting as a site distinct from aspirin in the synthetic cascade rather than administering a higher dose of the cyclooxygenase inhibitor. We hypothesized that if such a combined approach resulted in further inhibition of platelet thromboxane synthesis *in vivo*, redirection of platelet endoperoxides, which would accumulate after thromboxane synthase inhibition, might lead to an increase in vascular prostacyclin formation.^{22,23}

We used the excretion of 2,3-dinor-thromboxane B_2 as an index of endogenous thromboxane biosynthesis. This compound has been shown to be the major urinary metabolite of systemically administered thromboxane B_2 in man.²⁴ Several lines of evidence indicate that urinary 2,3-dinor-thromboxane B_2 is largely derived from platelet thromboxane synthesis. First, the pattern of inhibition and recovery of dinor thromboxane after administration of pharmacological inhibitors of thromboxane synthesis is consistent with a predominantly platelet origin of this metabolite. Thus, chronic administration of low-dose aspirin (20 mg daily⁶ and 20 mg twice a day¹⁷) has been shown in two studies in healthy volunteers to cause marked depression of 2,3-dinor-thromboxane excretion, by 85% and 70%, respectively. This occurred in the absence of any significant effect on prostacyclin biosynthesis that provides a marker of extraplatelet cyclooxygenase activity. Similarly, urinary thromboxane B_2 , which, in contrast to the dinor metabolite, is believed to be principally of renal origin under physiologic conditions in humans also remains unaltered following low-dose aspirin administration despite almost maximal inhibition of platelet thromboxane formation *ex vivo*.²⁵ The importance of these observations is that they address the possibility that the platelet represents a source of cyclooxygenase that is extremely sensitive to inhibition by aspirin but that contributes little to the concentration of 2,3-dinor-thromboxane B_2 in urine. Such a phenomenon would explain a discrepancy between the effects of single, low doses of aspirin (eg, 120 ng) on metabolite excretion and serum thromboxane B_2 , such as that illustrated in Fig 3. However, the marked depression of urinary 2,3-dinor-thromboxane B_2 that can be achieved without evidence of inhibition of prostaglandin formation by tissues other than the platelet *in vivo* during chronic administration of low-dose aspirin, renders unlikely such an explanation of our data. Finally, the recovery of dinor thromboxane excretion after irreversible acetylation of platelet cyclooxygenase by aspirin corresponds to platelet life span,^{16,17} while recovery is much more rapid after reversible enzyme inhibition induced by thromboxane synthase inhibitors⁴ or indomethacin.¹⁶ The results reported in this study also show recovery of 2,3-dinor-thromboxane B_2 occurring within 24 hours after the synthase inhibitor, but no recovery during the same observation period after aspirin administration.

A second line of evidence consistent with the platelet being the major source of urinary 2,3-dinor-thromboxane B_2 derives from the measurement of this metabolite in a large number of healthy volunteers as well as in diverse human

syndromes in which platelet activation in vivo has been documented. Over a wide range of platelet counts we have demonstrated a significant correlation between platelet number and dinor metabolite excretion in this study. Of particular interest is the distinction between reduced metabolite excretion in those subjects with a low platelet count as a result of decreased platelet production, and the marked elevation in 2,3-dinor-thromboxane B_2 in the two patients with thrombocytopenia due to increased peripheral destruction of platelets. Enhanced thromboxane synthesis has also been described in association with evidence of platelet activation in vivo in patients with severe peripheral vascular disease,⁴ unstable angina,¹⁹ and systemic sclerosis with Raynaud's phenomenon.⁵ Indeed, that it may be a highly sensitive index of platelet activation in vivo is suggested by the significant elevation is apparently healthy chronic smokers.¹⁷

Finally, inhibition of platelet activation in vivo, using measures that do not directly inhibit the thromboxane biosynthetic pathway, significantly reduces dinor thromboxane excretion. For example, infusion of prostacyclin to patients with thrombotic thrombocytopenic purpura results in a striking reduction in metabolite excretion.²⁶ Consistent with the hypothesis, cessation of smoking is followed by a corresponding reduction in dinor thromboxane.¹⁷ In addition, in patients with severe atherosclerosis and platelet activation in vivo, dietary supplementation with eicosapentaenoate markedly depressed excretion of the dinor metabolite.²⁷

Thus, if the assumption that the platelet is the predominant (although not only) source of the dinor metabolite in urine is valid, a progressive fall in excretion of this metabolite would be likely to reflect progressive inhibition of platelet thromboxane formation in vivo. The results of the present study would then indicate that inhibition of the capacity of platelets to generate thromboxane ex vivo may prove misleading as a guide to the inhibition of actual thromboxane biosynthesis by platelets and hence, thromboxane-dependent platelet activation in vivo. Inhibition of the final 6% of platelet capacity corresponded to inhibition of the final 72% of thromboxane formation in vivo (after aspirin 120 mg). The functional implications of this observation are illustrated by evidence that even a residual 10% capacity to generate thromboxane appears to be enough to fully sustain thromboxane-dependent platelet activation.⁷ Similarly, by increasing the inhibition of platelet thromboxane formation ex vivo from 95% to 99%, an apparently minimal increase, it is possible to achieve further measurable inhibition of platelet

function in normal volunteers, as assessed both by platelet aggregation ex vivo and the bleeding time.⁶ While these observations are not likely to be of clinical importance in healthy individuals, they may be extremely important when stimuli to cause platelet activation are present. The results of the present study indicate that thromboxane biosynthesis may remain markedly enhanced, despite substantial, but incomplete (eg 90%) inhibition of serum thromboxane B_2 .

The changes that we observed in prostacyclin biosynthesis provide further evidence that excretion of 2,3-dinor-thromboxane B_2 in the present study was derived predominantly from platelets. Aspirin 120 mg caused a transient depression in excretion of the prostacyclin metabolite, 2,3-dinor-6-keto-PGF_{1 α} . This is compatible with rapid de novo synthesis of endothelial cyclooxygenase following aspirin exposure, as has previously been shown to occur in cultured endothelial cells.²⁸ However, it contrasts with the more prolonged depression of prostacyclin metabolite excretion observed following chronic administration of much higher doses of aspirin than used in the present study.⁶ Interestingly, such a dose-dependent difference in recovery has also been shown to occur ex vivo in patients who have been receiving aspirin.^{29,30} By contrast, the thromboxane synthase inhibitor resulted in a significant increment in prostacyclin biosynthesis, consistent with utilization of accumulated platelet endoperoxides by vascular prostacyclin synthase.^{22,23,31} Addition of the thromboxane synthase inhibitor abolished the depression of prostacyclin biosynthesis seen when aspirin was administered alone. It is attractive to speculate that this may reflect reorientation of accumulated endoperoxides resulting from additional inhibition of platelet thromboxane formation in vivo, in this case occurring at the level of thromboxane synthase.

Measurements of the platelet capacity to generate thromboxane B_2 ex vivo are frequently employed as a guide to the adequacy of dosing with platelet-inhibitory drugs.³² The results of the present study suggest that biosynthesis of thromboxane is maintained to a substantial degree, unless >95% inhibition of thromboxane generation ex vivo is achieved. Lesser degrees of inhibition would not be expected to substantially influence thromboxane-dependent platelet activation in vivo.

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Is Heparin Administration Necessary During Induction Chemotherapy for Patients With Acute Promyelocytic Leukemia?

By Mark A. Goldberg, David Ginsburg, Robert J. Mayer, Richard M. Stone, Maryellen Maguire, David S. Rosenthal, and Joseph H. Antin

The role of heparin in the treatment of the disseminated intravascular coagulation (DIC) associated with acute promyelocytic leukemia (APL) remains unclear. Between 1974 and 1985, we treated 27 patients with APL using four different chemotherapeutic regimens; 23/27 (85%) had evidence of DIC either at presentation or following the initiation of induction chemotherapy. The coagulopathy was treated primarily with fresh frozen plasma and platelet transfusions; only 2/27 (7%) patients received heparin. Twenty of 27 patients (74%) entered complete remission. Major bleeding or thrombotic complications occurred in 5/27 patients (19%), but 2 of these 5 patients presented

after hemorrhage had already occurred. None of the 5 patients with bleeding or thrombosis entered complete remission. All of the hemorrhagic complications due to DIC in our study occurred before 1979, which may reflect changes in the management of leukemic patients. This observation emphasizes the risks inherent in the use of historical controls in this population. In conclusion the DIC associated with APL can be successfully treated with intensive blood product support without the use of heparin.

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IN contrast to other forms of acute nonlymphocytic leukemia, acute promyelocytic leukemia (APL) is frequently associated with a bleeding diathesis, initially described by Hillestad in 1957.¹ He reported three patients with a rapidly fatal acute leukemia in whom the predominant cell type was an abnormal promyelocyte. All three patients had a severe coagulopathy characterized by thrombocytopenia, hypofibrinogenemia, prolonged bleeding times and thrombin times, and a decreased clot lysis time. While there is some evidence to suggest that the coagulopathy represents primary fibrinolysis,²⁻⁶ it is now generally accepted that the bleeding tendency is primarily the consequence of disseminated intravascular coagulation (DIC) occurring in response to procoagulants released from the abnormal promyelocytic granules.⁵⁻¹¹ As a result, the use of heparin to treat or prevent this form of DIC in patients with APL was introduced by several investigators^{2,12-19} and has become accepted as a standard method of practice,^{20,21} despite the absence of a prospective randomized trial documenting its efficacy. We have been reluctant to administer heparin to thrombocytopenic, critically ill patients without unequivocal demonstration of its value. Consequently, during the past 11 years the majority of our patients (25/27) with APL have been managed with intensive fresh frozen plasma (FFP) and platelet support, and intensive chemotherapy without the concomitant use of heparin. The results of our experience and a review of the literature comprise this report.

METHODS

We retrospectively reviewed all cases of acute myelogenous leukemia diagnosed and treated at the Brigham and Women's Hospital and the Dana-Farber Cancer Institute between January 1974 and June 1985. During this period, 27/371 (7%) patients fulfilled the morphologic criteria of the French-American-British (FAB) classification²² for hypergranular APL (FAB M3 histologic subtype). Since 1981 cytogenetic studies were performed in the Dana-Farber Cancer Institute cytogenetics laboratory. The 27 patients were treated with various remission induction chemotherapy regimens, all of which contained cytosine arabinoside and an anthracycline (Table 1) as described previously.²³⁻²⁵

Complete remission (CR) was defined according to CALGB criteria as less than 5% blasts on bone marrow aspiration with evidence of maturation of all bone marrow cell lines and restoration of peripheral blood counts.²⁶ The diagnosis of DIC in this leukemic

population was defined as the presence of any two of the following abnormalities: (1) prothrombin time (PT) 3 or more seconds greater than control, (2) activated partial thromboplastin time (aPTT) 5 or more seconds greater than the upper limit of the normal range, (3) thrombin time (TT) prolonged by 3 or more seconds compared to control, (4) fibrinogen less than 150 mg/dL, and (5) fibrin degradation products (FDP) greater than or equal to 40 µg/mL. Survival was calculated from the date of diagnosis and was assessed by the life table analysis described by Peto et al.²⁷ The Mantel-Haenszel test was used to compare differences between different cohorts.²⁸

Coagulation parameters and complete blood counts were obtained at least once daily. All patients with thrombocytopenia, anemia, evidence of DIC, or clinical bleeding were treated with platelet and red blood cell transfusions and fresh frozen plasma. In general, in the absence of bleeding, platelet transfusions were administered when the platelet count fell to 20,000/µL or less and fresh frozen plasma (2 to 4 U/d) was given to maintain a plasma fibrinogen concentration of approximately 150 mg/dL or to correct the prothrombin time to less than 3 seconds beyond control. Heparin was neither routinely used for the treatment of DIC nor was it given prophylactically. Any decision concerning the initiation of heparin treatment in patients with laboratory evidence of DIC was left to the discretion of the responsible physician. Induction chemotherapy was begun within 48 hours after diagnosis, even if DIC were present.

RESULTS

Patient characteristics. The characteristics of the 27 patients with APL are summarized in Table 2. The median

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Table 1. Induction Regimens

Regimen (23-25) (Dates employed)	n	
COD (1970-1974)	4	cytosine arabinoside 2 mg/kg/d IV bolus $\times$ 3-6 d daunorubicin 1 mg/kg/d IV bolus $\times$ 3-6 d vincristine 1 mg/m ² IV bolus $\times$ 1 d
DAC I (1974-1977)	3	cytosine arabinoside 2 mg/kg/d continu- ous IV infusion $\times$ 5-7 d daunorubicin 1 mg/kg/d IV bolus $\times$ 3 d
VAPA 10 (1977-1980)	6	cytosine arabinoside 100 mg/m ² d continu- ous IV infusion $\times$ 7 d doxorubicin 30 mg/m ² d IV bolus $\times$ 3 d vincristine 1 mg/m ² IV bolus days 1,8 methylprednisolone 40 mg/m ² d IV bolus days 1-5
DAC II (1980-1985)	14	cytosine arabinoside 200 mg/m ² d con- tinuous IV infusion $\times$ 7 d daunorubicin 45 mg/m ² d IV bolus $\times$ 3 d

age was 37; there were 14 males and 13 females. Generally, patients were severely thrombocytopenic and anemic. As previously reported,⁸ leukopenia was commonly observed; the median white blood cell (WBC) was 1,850/mm³. Cytogenetic studies were performed on marrow specimens from 8 patients; 6 of these 8 had the 15:17 translocations that are characteristic of APL.²⁹ No other cytogenetic abnormalities were observed. Twenty-three of the 27 (85%) patients had DIC at diagnosis or developed DIC with institution of chemotherapy but only 2 of the 27 patients received heparin. The incidence of specific coagulation abnormalities is detailed in Table 2.

Treatment of coagulopathy. In order to determine the transfusion requirements of patients with DIC who were not treated with heparin, we analyzed the records of the group of patients who were treated without heparin on the VAPA and DAC II protocols (since after 1979 there was more uniform availability of platelet concentrates in our institutions).

Table 2. Patient Characteristics

Number of patients	27
Median age (range)	37 (20-69)
Sex (M/F)	14/13
Median blood counts (range)	
Hematocrit	28.5% (17-41)
WBC (/mm ³)	1850 (330-41,000)
Platelets ($\times 10^3$ /mm ³)	18 (5-161)
Number of patients with DIC	23
Incidence of abnormal coagulation parameters*	
PT	16 (70%)
aPTT	4 (17%)
TT	15 (68%)†
fibrinogen	21 (91%)
FDP	21 (96%)†
Treatment or prophylaxis of DIC	
Heparin, FFP, and platelets	2
Platelets and/or FFP	25

*Percentage of patients with DIC who demonstrated the abnormal coagulation test is shown in parentheses. The definition of abnormal values is described in Methods.

†Data was only available on 22 patients with DIC.

Fifteen of these 18 patients met our criteria for DIC, and we have complete transfusion records on 14 of the 15. The median duration of DIC as defined above was four days (range one to ten days). The FDPs were elevated (≥ 40 μ g/mL) for a median of six days (range zero to ten days), while the plasma fibrinogen was <150 mg/dL for a median duration of four days (range one to ten days). Fresh frozen plasma was often administered at times when patients did not meet our strict criteria for DIC but had elevated FDPs or low fibrinogens. Consequently we divided the total number of units of FFP and platelets administered per patient by the duration of DIC or seven days, whichever was longer. That number provides an estimate of the daily blood product requirements during the period of highest risk for the development of DIC. The patients received a median number of 1.9 units of FFP/d (range 0.3 to 6.3 U/d) and 6 units of platelets/d (range 0 to 11 U/d). The coagulation parameters and response to therapy of a representative patient is shown in Fig 1.

Hemorrhagic and thrombotic complications. Five of the 27 patients (19%) had major bleeding or thrombotic complications. Two of these 5 patients had intracranial hemorrhages and were moribund when we first evaluated them, while 2 additional patients developed intracranial hemorrhages while receiving induction chemotherapy. It was noteworthy that only 4 patients had prolonged activated partial thromboplastin times and 3 of these 4 died of intracranial hemorrhage. Hepatic vein thrombosis and pulmonary hemorrhage occurred in the fifth patient during the second course of induction therapy. However, postmortem examination

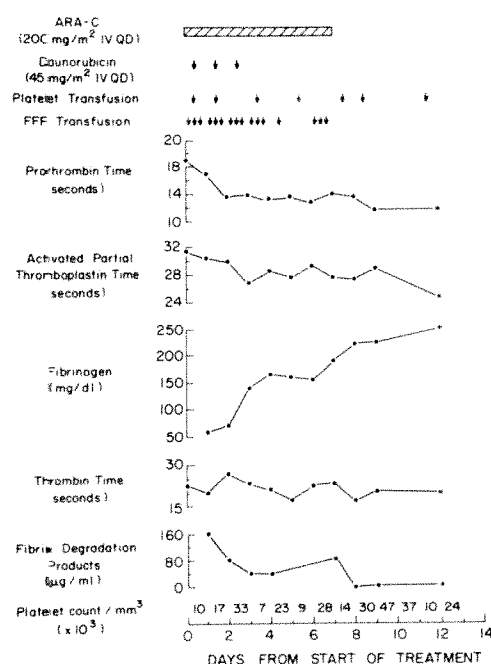


Fig 1. Response to therapy of a representative patient. This patient had abnormalities of all five coagulation parameters at diagnosis. Institution of chemotherapy and blood product support resulted in rapid amelioration of the DIC. Each arrow indicating a platelet transfusion represents 6 to 10 units of platelets.

revealed hepatic venoocclusive disease (VOD) without laboratory or pathologic evidence of DIC.

Remission and survival data Twenty of the 27 patients (74%) entered CR. The median follow-up of the group was 503 days (range 1 to 2706 days) and the probability of survival at 3 years was $38 \pm 11\%$ (Fig 2). If the patients who achieved a complete remission were examined separately, the median follow-up was 632 days (range 151 to 2706 days) and the probability of survival at 3 years was $51 \pm 13\%$ (Fig 2).

DISCUSSION

The etiology of the hemorrhagic diathesis associated with APL has been the subject of much study. Several investigators have demonstrated an increased rate of fibrinogen catabolism in patients with APL.³⁰⁻³³ However, the relative contributions of primary fibrinolysis and DIC to the coagulopathy of APL are controversial. In a review of the literature, Groopman and Ellman⁸ found that measurements of plasminogen and plasmin levels, as well as clot lysis and euglobulin lysis times yielded variable results and generally had not provided convincing evidence of systemic fibrinolysis. Evidence in support of primary fibrinolysis includes the report by Keene et al³ of three patients with APL treated with epsilon-amino caproic acid (EACA). Although the bleeding in these patients ceased and they all entered CR, the absence of detailed data concerning coagulation studies makes the contribution of EACA difficult to interpret. Bennett et al⁴ also proposed a role for primary fibrinolysis in the hemorrhagic diathesis seen in APL based on their observation that transfused ⁵¹Cr-labeled autologous platelets had a normal life span in a patient with APL while the half life of ¹²⁵I-labeled fibrinogen was markedly decreased.

Several lines of investigation have pointed to a major role for DIC in the coagulopathy of APL. In 1973, Gralnick and Abrell⁵ demonstrated significant procoagulant properties associated with the lysates of leukocytes obtained from patients with APL. The procoagulant activity was further localized to the granular fraction of these leukocytes and was not present in granular fractions obtained from normal controls or patients with other forms of acute nonlymphocytic leukemia. They also demonstrated a less marked, but significant increase in fibrinolytic activity present in the granular fraction in 3 of 4 patients with APL. Furthermore, a response to heparin with normalization of clotting parameters and clinical cessation of bleeding suggested that DIC played a predominant role in the bleeding diathesis of APL.¹² Bauer and Rosenberg¹¹ provided direct evidence that patients with APL generate excessive amounts of thrombin within the vascular system. They employed sensitive radioimmunoassays to study the levels of prothrombin activation fragment, F_{1+2} , thrombin-antithrombin complex (TAT), and fibrinopeptide A (FPA) in patients with APL undergoing intensive chemotherapy. They showed an excellent correlation between elevations in F_{1+2} , TAT, and FPA. Finally, postmortem findings consistent with DIC in patients with APL have been frequently reported.^{9,10,12,34}

Baker et al³⁵ first reported the use of heparin in 1964 in the treatment of a hemorrhagic diathesis in a patient with AML who had 24% promyelocytes with some atypical forms in his

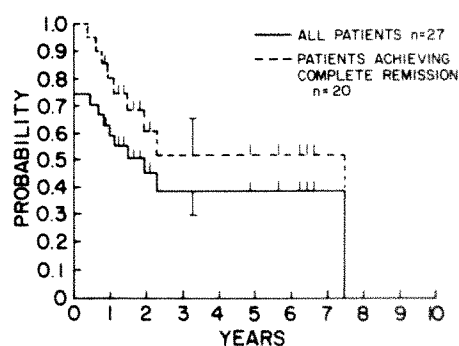


Fig 2. Life table analysis of overall survival of patients with APL; All patients (solid line), and all patients who achieved a complete remission (interrupted line). The bars indicate the standard error at 3 years. Tic marks indicate patients who are currently alive.

bone marrow aspirate. The patient had gross clinical bleeding unresponsive to EACA. Initially low levels of fibrinogen, factor V, and plasminogen all increased after institution of heparin therapy. However, the patient subsequently died and postmortem examination showed findings consistent with widespread DIC and leukemic infiltration. In 1972, Gralnick et al¹² reported four patients with APL and DIC, of whom three were treated with heparin and one with platelet transfusions, fresh frozen plasma, and fibrinogen. All four patients died; autopsy revealed that 3 of the 4 had persistent leukemia and all had evidence of thrombosis and/or hemorrhage. However, the three patients treated with heparin did have a marked improvement in coagulation abnormalities associated with decreases in clinical bleeding.

The goal of heparinization in patients with APL is to inhibit DIC by stopping the accelerated fibrin formation and associated secondary fibrinolysis. To examine the kinetics of fibrin formation in such patients, Bauer and Rosenberg¹¹ measured the formation of FPA in patients receiving heparin. The only patient to have a significant reduction in FPA production received 1,000 u/m²/hr of heparin. This patient also had a doubling of the aPTT while receiving heparin. Hence, both normal as well as pathologic coagulation may have been inhibited. Clearly, in a patient with a platelet count in the range of 20,000/mm³, heparinization is not without significant risks.

The coagulopathy commonly observed in patients with APL at the time of diagnosis is thought to reach its maximal intensity shortly after the initiation of chemotherapy at which time the destruction of leukemic cells results in the release of procoagulant-rich granules. Once significant cytoreduction has been achieved, the stimulus for the coagulopathy diminishes and clotting parameters return to normal. Therefore, our efforts in addressing the DIC associated with APL have focused on the rapid removal of the cause of DIC (ie, the abnormal promyelocytes) by the prompt initiation of chemotherapy. We support such patients with judicious platelet and fresh frozen plasma transfusions until the DIC has resolved (Fig 1). Our patients received doses of FFP and platelets which are easily obtained by most blood banks. Heparin was used in the management of only 2 (7%) of our 27 patients with no patients being so treated during the last 4

years. Our overall complete remission rate of 74% compares favorably with results reported by others, both in studies in which heparin was and was not used (Table 3). There were 4 (15%) hemorrhagic deaths in our patient cohort, all of which were due to intracranial hemorrhage; 2 of these 4 hemorrhagic deaths could not have been prevented with heparin since the patients presented with major intracranial catastrophes at the time of their diagnoses. Although the aPTT was the least sensitive measure of the presence of DIC in this patient population, elevations of the aPTT at presentation appeared to be associated with severe hemorrhagic complications. Three of the 4 patients who had prolonged aPTT's died of intracranial hemorrhage. While the number of patients is too small for reliable statistical inferences, perhaps patients with a prolonged aPTT reflect a subgroup with a worse prognosis.

The impact of heparin on the management of patients with APL remains unclear. A compilation of reported series of patients with APL reveals no statistically significant benefit for the use of the anticoagulant with a 19% incidence of hemorrhagic deaths being associated with the use of heparin compared to a 28% death rate without heparin ($P > 0.05$, Table 4). The complete remission rate of these combined series using heparin was 63% in contrast to 54% without heparin ($P > 0.1$, Table 3). It is noteworthy that the majority of patients comprising the "nonheparin" group were treated more than 10 years ago. Seventeen (85%) of the 20 patients we have treated during the past 6 years have entered complete remission without any hemorrhagic deaths. This higher probability of remission is consistent with the generally accepted improved response rate observed in patients with acute nonlymphocytic leukemia during the last decade.³⁷ Such an improvement may be attributable not only to more intensive chemotherapeutic regimens, but also to the increased availability of blood product support and improvements in antimicrobial agents. The introduction of prophylactic heparin therapy into APL induction regimens temporally corresponded to these advances in care. Since the

Table 3. Effect of Heparin on the CR Rate of Adults With APL (anthracycline-containing regimens only)

Reference	Years	With Heparin		Without Heparin	
		n	# CR (%)	n	# CR (%)
Drapkin et al ¹³ *	1974-1976	9	7	8	2
Ruggero et al ³⁶ *	1972-1976	0	—	13	7
Daly et al ¹⁴ *	1973-1979	10	8	5	3
Collins et al ¹⁵ †	1973-1975	7	5	0	—
Bernard et al ¹⁶ *	1968-1971	9	3	35	16
Cordonnier et al ¹⁷ *	1972-1982	57	30	0	—
Cunningham et al ¹⁸ *	1974-1983	54	41	3	2
Kantarjian et al ¹⁹ †	1973-1984	31	18	28	14
Present Study†	1974-1985	2	1	25	19
Total		179	113 (63%)	117	63 (54%)

$P > 0.10$ ‡

*These patients also received platelet transfusions.

†These patients received platelets and fresh frozen plasma; Collins et al also used cryoprecipitate.

‡Mantel-Haenszel test.

Table 4. Effect of Heparin on the Incidence of Hemorrhagic Deaths in Adults With APL During Induction Chemotherapy (anthracycline-containing regimens only)

Reference	With Heparin		Without Heparin	
	n	Hemorrhagic Deaths	n	Hemorrhagic Deaths
Drapkin et al ¹³ *	9	0	8	5
Ruggero et al ³⁶ *	0	—	13	4
Daly et al ¹⁴ *	10	1	5	1
Collins et al ¹⁵ †	7	1	0	—
Bernard et al ¹⁶ *	9	4	35	9
Cordonnier et al ¹⁷ *	57	15	0	—
Cunningham et al ¹⁸ *	54	7	3	1
Kantarjian et al ¹⁹ †	31	6	28	9
Present Study†	2	0	25	4‡
Total	179	34 (19%)	117	33 (28%)

$P > 0.05$ §

*These patients also received platelet transfusions.

†These patients received platelet transfusions and fresh frozen plasma; Collins et al also used cryoprecipitate.

‡0/20 hemorrhagic deaths during the past six years.

§Mantel-Haenszel test.

apparent benefit of such anticoagulation was only appreciated by a comparison with historical experience, it is possible that the more recently observed improvement in outcome is in fact due to these other advances in care and not due to heparin therapy. Finally, in all recently published studies in which heparin has been used, patients were concomitantly treated with platelet concentrates, at times in association with fresh frozen plasma. Such platelet concentrates are known to contain significant amounts of coagulation factors.³⁸ Therefore, it is conceivable that much of the improvement in clotting parameters and control of clinical bleeding observed in patients with APL who were given heparin might actually have been the result of the simultaneous administration of platelet concentrates and fresh frozen plasma.

We conclude that the coagulopathy associated with APL can be successfully managed with intensive chemotherapy and blood product support without the routine use of heparin. The necessity for the inclusion of prophylactic heparin therapy into the remission induction regimens of all patients with APL has not been proven, and the impact of such anticoagulant therapy remains unclear. To clarify the relative values of fresh frozen plasma and platelet transfusion and heparin prophylaxis, a prospective randomized trial would be required.

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Thrombocytopoietic Response to Immunothrombocytopenia in Nude Mice

By Shirley Ebbe, Jack Levin, Kathleen Miller, Tamlyn Yee, Francine Levin, and Elizabeth Phalen

Thrombocytopoiesis was evaluated in T cell-deficient nu/nu mice and in T cell-replete nu/+ controls to determine if abnormalities would be associated with the deficiency of T cells. Mice were studied in the unperturbed steady state and after acute immunothrombocytopenia was induced by an injection of guinea pig antmouse platelet serum (APS). The state of thrombocytopoiesis was determined from platelet counts, megakaryocyte size, megakaryocyte number, and numbers of Meg-CFC. Splenic lymphocytes were evaluated by responses to the mitogens bacterial lipopolysaccharide (LPS), phytohemagglutinin (PHA), and concanavalin A (Con A). Hematocrits, reticulocyte counts, leukocyte counts, marrow cellularity, GM-CFC, and BFU-E also were measured. Steady state thrombocytopoiesis was identical in nu/nu and nu/+ mice. In response to an injection of APS, acute thrombocytopenia was followed by macromegakaryocytosis and rebound thrombocytosis in mice of both genotypes. Splenic Meg-CFC increased in nude mice after APS or an injection of normal guinea pig

serum (NGpS), and splenic GM-CFC increased after APS. Neither Meg-CFC nor GM-CFC increased in the spleens of nu/+ mice, but they showed early transient increases in bone marrow that did not occur in nu/nu mice. Sporadic, but weak, mitogenic responses to PHA or Con A were occasionally observed with nu/nu spleen cells, but these did not correlate with the state of thrombocytopoiesis. The results demonstrated that platelet production was normal in nu/nu mice and that megakaryocytopoiesis and platelet production responded to the stimulus imposed by acute immunothrombocytopenia. Increases in megakaryocyte size and platelet production occurred independently of changes in numbers of Meg-CFC, GM-CFC, or BFU-E. A normal complement of T cells appears to be unnecessary for normal platelet production and its augmentation in response to the stimulus of acute immunothrombocytopenia in vivo.

This is a US Government work. There are no restrictions on its use.

THE rate of platelet production is appropriately adjusted in response to experimental perturbations of the platelet count. Therefore, it can be concluded that thrombocytopoiesis is regulated. Several levels of platelet precursors have been shown to respond to the stimulation of thrombocytopoiesis that is produced in experimental animals by injection of heterologous antiplatelet serum (APS) to induce thrombocytopenia. Alterations of recognizable megakaryocytes and their immediate precursor cells tend to occur promptly after induction of thrombocytopenia,¹⁻³ but responses of less differentiated cells, such as megakaryocyte colony-forming cells (Meg-CFC), are generally relatively delayed.⁴⁻⁶ The observations are consistent with multiple levels of megakaryocytic regulation in which more mature cells respond to a platelet-dependent regulatory mechanism, ie, thrombopoietin, and less mature cells respond to other, as yet unidentified, mechanisms.⁷ However, cellular interactions

that may be responsible for control of thrombopoietin production or mediation of other thrombocytopoietic regulatory functions have not been identified.

Dual regulation also has been proposed for growth of Meg-CFC in vitro.⁸ Both a colony-stimulating factor that promotes cell proliferation and a potentiator for maturation have been reported to be necessary.⁸ However, growth of megakaryocyte colonies, as well as cells that form colonies of other types of hematopoietic cells, can also be induced by the conditioned medium that is produced by spleen cells when they are exposed to a T cell mitogen, and this medium appears to contain all growth factors necessary for proliferation and maturation of megakaryocytes in vitro.^{9,10} Because of the potent effect of T cell products on Meg-CFC in vitro and the occasionally associated occurrence of platelet and immunological abnormalities in vivo, it has been proposed that T cells may play a thrombocytopoietic regulatory role in vivo.^{11,12} The possibility also has been suggested that immunothrombocytopenia, induced by APS, might result in changes in Meg-CFC that were more dependent on immunological stimulation of T cells than on the thrombocytopenia per se.¹³

The present experiments were based on the concept that if T cells are important for the regulation of platelet production or for some aspects of the responses to APS, abnormalities of thrombocytopoiesis or its response to immunothrombocytopenia might be found in T cell-deficient nude (nu/nu) mice. Athymic mice have been reported to have normal platelet and megakaryocyte counts,¹⁴ but their ability to increase platelet production in response to stimulation has not been reported.

MATERIALS AND METHODS

Mice. Athymic nude (nu/nu) mice were studied, and heterozygous hirsute nu/+ mice were used as controls. Mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass) and from NCI-Frederick Cancer Research Facility (Frederick, Md). Nu/nu mice were housed, 5/cage, in a protective environment (Airo Clean Engineering Inc., Broomall, Pa); nu/+ mice were kept in a

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standard mouse room. At the time of study, the mice were about 12 weeks old; each mouse was anesthetized with ether, a sample of cardiac blood was drawn, and the mouse was killed by cervical dislocation.

Blood and bone marrow cell counts. The cardiac blood samples were anticoagulated with dry K_2EDTA . Platelet counts were done by phase microscopy.¹⁵ Microhematocrits were determined with a Drummond centrifuge (Drummond Scientific, Broomall, Pa). Reticulocytes were counted by microscopy after staining with new methylene blue.¹⁶ Leukocytes were counted with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla) at a 1:500 dilution in Isoton and Zapoglobin (Coulter).

Bone marrow was flushed from each tibia with 1 mL of 1% Na_2EDTA in saline. Total cells were counted by Coulter counter at a 1:500 dilution in Isoton and Zapoglobin. Megakaryocytes were counted by microscopy with a hemacytometer, after addition of 0.1 volume of new methylene blue; duplicate counts were done on each tibia, and the average number for both tibiae was calculated.

Megakaryocyte size. Smears of bone marrow were made from split femurs with a 000 paint brush and then stained with Wright's and Giemsa stains. Stage III megakaryocytes were identified by microscopy based on the morphological characteristics of azurophilic granules throughout the cytoplasm and low nucleus:cytoplasm ratio. Areas of stage III cells that had intact cytoplasmic borders were measured microscopically with a Zeiss MOP-3 digital image analysis system (Carl Zeiss, Oberkochen, W. Germany). Sizes were recorded as "units"; each unit was equal to 1 mm^2 of the measuring tablet. A stage micrometer with divisions equal to 10μ was used to determine that the linear magnification was $1230\times$. Based on a circular shape for the cells (a reasonable approximation for most megakaryocytes) 1000 "units" corresponded to an area of $660\mu^2$ in the bone marrow smear. Areas of 50 megakaryocytes were measured for each mouse, and the average area was calculated. This average was then used to calculate average areas for each treatment group; that is, each mouse was represented in the final calculations by one number, not 50 numbers.

Guinea pig sera. Blood was collected, by cardiac puncture, from untreated guinea pigs under ether anesthesia. After it clotted, normal guinea pig serum (NGpS) was prepared by centrifugation and stored at -20°C . It was diluted 1:4 with saline, and 0.1 mL was injected intraperitoneally into each mouse treated with NGpS.

Guinea pig antimouse platelet serum (APS) was prepared by injection of guinea pigs with mouse platelets ($\sim 3 \times 10^9$ platelets/injection) suspended in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich) and saline. The first injection was given into the foot pads and subcutaneously; 3 subsequent injections were given subcutaneously at 1, 2, and 4 weeks after the first. Immune serum was collected from cardiac blood 1 week after the final injection, and stored at -20°C . Prior to use, it was heated to 56°C for 30 minutes, absorbed 3 times with equal volumes of washed mouse red cells, and then again stored at -20°C . It was diluted 1:4 with saline, and 0.1 mL was injected intraperitoneally into each mouse treated with APS.

Megakaryocyte and granulocyte-macrophage colony-forming cells (Meg-CFC and GM-CFC). Single-cell suspensions were prepared from bone marrow and spleen as previously described.^{4,17} All cultures were performed in 35 mm diameter plastic Petri dishes (Falcon Laboratories, Oxnard, Calif) using a 0.3% agar medium which contained Dulbecco's modified Eagle's medium and 20% horse serum (Flow Laboratories, Inc., McLean, Va), prepared according to a modification of the method of Metcalf et al.⁹ To each culture dish, 0.1 mL of pokeweed mitogen-stimulated spleen-conditioned medium was added. Bone marrow cultures contained 25,000 or 50,000 cells/mL, and spleen cell cultures contained 0.5×10^6 or 1.0×10^6 cells/mL.

After seven days of incubation at 37°C in 100% humidity and 10% CO_2 in air, cultures were scored for colonies using an Olympus dissecting microscope (Olympus Corp., Lake Success, NY) with indirect lighting and $\times 35$ to 40 magnification. Aggregates of 50 or more cells were scored as colonies except for megakaryocytes, for which 3 or more cells were considered a colony. Data presented in this article were based on triplicate cultures of cells pooled from 2 mice for each experimental point in each experiment. Granulocyte-macrophage (GM) colonies and megakaryocyte (Meg) colonies were enumerated, based on their morphological characteristics. The number of Meg colonies was confirmed by drying the agar cultures in situ with a hot plate and hair drier, followed by staining of the dried cultures for acetylcholinesterase, as described previously.^{4,17} Colonies were considered megakaryocytic if at least 3 cells demonstrated the characteristic orange-brown color, indicative of acetylcholinesterase, in the cytoplasm. The total numbers of Meg-CFC and GM-CFC per organ (femur or spleen) were calculated from their respective frequencies in cultures and the total nucleated cell count per femur or spleen.

Erythroid burst-forming unit (BFU-E). Humeral bone marrow suspensions were prepared in RPMI 1640 (Gibco Laboratories, Santa Clara, Calif) supplemented with $50\mu\text{g/mL}$ gentamycin sulfate (Schering Corp, Kenilworth, NJ). Marrow was flushed from the bone using a 22 gauge needle, and single-cell suspensions were prepared by gently passing the cells through a 27 gauge needle. Cells were counted using a hemacytometer, and viability was determined by eosin exclusion. BFU-E were cultured in methylcellulose using a modification of the method of Stewart et al.¹⁸ The culture medium consisted of 0.8% methylcellulose (Dow Chemical, Walnut Creek, Calif), 1 mmol/L L-glutamine (Gibco), 10^{-4} mol/L mercaptoethanol (Sigma Chemical Co., St. Louis, Mo), $28\mu\text{g/mL}$ CaCl_2 (Sigma), 1% BSA (Fraction V, Miles Scientific, Naperville, Ill) which had been deionized and lyophilized according to the method of McLeod et al.,¹⁹ 30% fetal bovine serum (FBS; Flow), and alpha MEM (Gibco) containing $50\mu\text{g/mL}$ gentamycin sulfate (Schering). Human urinary erythropoietin (4.78 U/mg; kindly supplied by Dr Gisela Clemons) was added at a final concentration of 2.5 U/mL. Bone marrow cells were plated at a concentration of 2×10^5 cells/mL. Cultures were incubated at 37°C in 5% CO_2 in air with saturated humidity. Bursts were scored in unstained cultures after seven days using an inverted microscope at $40\times$ magnification. Erythroid bursts were identified by the presence of hemoglobin which gave a pink-orange-red color to the cells and by their colony morphology and individual cell size. Colonies with multiple large aggregates that contained >50 cells (usually >100) were scored as BFU-E. Data are presented as mean number of BFU-E $\pm$ SEM for at least 6 cultures of pooled marrow from 3 mice/group.

Mitogen assays. Spleen cell suspensions were prepared in RPMI 1640 (Gibco) supplemented with $50\mu\text{g/mL}$ gentamycin sulfate (Schering). Cell suspensions were made by teasing cells loose from the capsule with an 18 gauge needle. Single-cell suspensions were made by passing the cells several times through the tip of a Pasteur pipette followed by passage through a fine mesh nylon sock to remove the large clumps. The cells were counted using a hemacytometer, and viability was determined by eosin exclusion. Spleen cells were cultured in RPMI 1640 (Gibco) that contained 5% FBS (HyClone Laboratories, Logan Ut; a lot screened for low background response), 1 mmol/L sodium pyruvate (Gibco), 1% nonessential amino acids (Gibco), $50\mu\text{g/mL}$ gentamycin sulfate (Schering), and $5 \times 10^{-5}\text{ mol/L}$ mercaptoethanol (Sigma). Cells were cultured in quadruplicate in 96 well microtitre plates (Falcon) in a total volume of 0.2 mL/well containing 5×10^5 spleen cells and $2.5\mu\text{g/mL}$ phytohemagglutinin (PHA, Difco), $2.5\mu\text{g/mL}$ concanavalin A (Con A) (Miles), or $25\mu\text{g/mL}$ lipopolysaccharide (LPS; Difco). Cultures were incubated at 37°C in a humidified atmosphere.

containing 5% CO₂. After 48 hours, 1 microcurie of tritiated thymidine (³HTdR; New England Nuclear, Boston, Mass) was added to each well; 16 hours later the cultures were harvested and cell radioactivity determined. Samples were harvested onto glass fibre filters using an automated sample harvester (Dynatech Laboratories, Inc., Alexandria, Va), and incorporated radioactivity was measured using a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif). The results are expressed as the mean counts per minute (cpm) of quadruplicate microwells $\pm$ SEM.

Statistical analysis. The significance of differences between mean values for different groups was determined with Student's *t* test. Only cases with *P* < 0.05 are reported as significantly different.

RESULTS

In the present experiments, two approaches were utilized to determine if regulation of thrombocytopoiesis was normal or abnormal in *nu/nu* mice. The first was to compare hematological data in untreated *nu/nu* mice with those of untreated *nu/+* controls. The second was to compare the responses of the two genotypes to acute thrombocytopenia produced by a single injection of APS.

Blood and marrow findings in untreated *nu/nu* and *nu/+* mice are presented in Table 1. Platelet counts, numbers of tibial megakaryocytes, sizes of mature megakaryocytes, and leukocyte counts were identical in both. The average hematocrit was slightly, but significantly, lower in *nu/nu* than in *nu/+* mice, but reticulocyte percentages were the same. Total tibial cellularity was lower in *nu/nu* than in *nu/+*.

Figure 1 shows data that confirm the T cell-deficient state of spleen cells from untreated nude mice. Spleen cells from *nu/+*, but not those from *nu/nu*, mice were stimulated to incorporate ³HTdR by incubation with the T cell mitogens PHA or Con A. Spleen cells from both genotypes responded equally to the B cell mitogen LPS.

Figure 2 shows blood platelet counts after an injection of APS, NGpS, or saline. Severe thrombocytopenia was present four hours after APS. Extensive cutaneous petechiae were visible on the hairless *nu/nu* mice. The subsequent recovery of platelet counts was similar in both genotypes. Significant rebound thrombocytosis occurred on days 5 and 6 with platelet counts of 146% to 193% of controls injected with NGpS. Day 6 platelet counts were significantly higher in

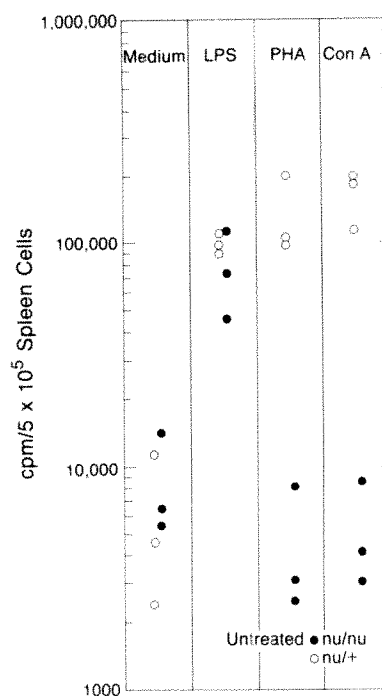


Fig 1. Tritiated thymidine incorporation into 5×10^5 spleen cells from untreated *nu/nu* and *nu/+* mice, assayed after culture in medium alone (background) or in the presence of a mitogen. Each point represents the mean counts per minute (cpm) for quadruplicate cultures.

nu/nu than *nu/+* mice injected with APS, but both genotypes showed thrombocytosis relative to controls injected with NGpS.

Average sizes of stage III megakaryocytes after an injection of APS, NGpS, or saline are presented in Fig 3. There was an abrupt increase in size on day 2 after APS to 141% to 161% of the size in controls injected with NGpS, after which sizes returned to normal.

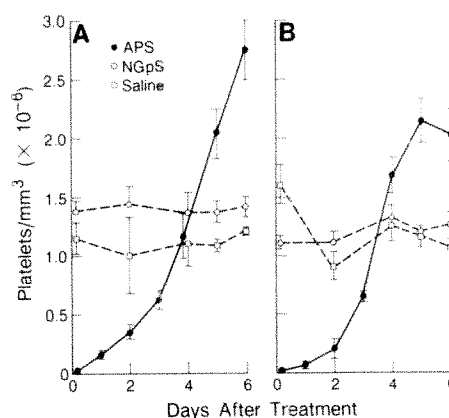


Fig 2. Platelet counts of (A) *nu/nu* and (B) *nu/+* mice after a single injection of APS, NGpS, or saline given at time zero. Values for APS and NGpS are the mean $\pm$ 1 SEM for 4 to 14 mice. Values for saline represent 3 to 4 mice. Platelet counts of *nu/nu* and *nu/+* mice injected with APS were significantly greater than controls injected with NGpS on days 5 and 6. On day 6, platelet counts of *nu/nu* mice injected with APS were higher than those of APS injected *nu/+* mice.

Table 1. Hematologic Data of Untreated Mice

	<i>nu/nu</i>	<i>nu/+</i>
Number	26	26
Platelets/mm ³ ($\times 10^{-6}$)	1.376 $\pm$ 0.050§	1.337 $\pm$ 0.038
Megakaryocytes/tibia ($\times 10^{-3}$)	8.0 $\pm$ 0.7	8.7 $\pm$ 0.6
Megakaryocyte size (units)‡	1553.0 $\pm$ 49.1	1506.9 $\pm$ 41.8
Hematocrit (%)	45.4 $\pm$ 0.4	46.6 $\pm$ 0.3*
Reticulocytes (%)	1.6 $\pm$ 0.2	1.4 $\pm$ 0.1
Leukocytes/mm ³	2059 $\pm$ 198	1800 $\pm$ 179
Cells/tibia ($\times 10^{-6}$)	11.6 $\pm$ 0.3	13.5 $\pm$ 0.5†

**P* < 0.025. Significance of difference between *nu/nu* and *nu/+*. Unmarked, no difference.

†*P* < 0.005. Significance of difference between *nu/nu* and *nu/+*.

‡Stage III megakaryocytes.

§Mean $\pm$ 1 SEM.

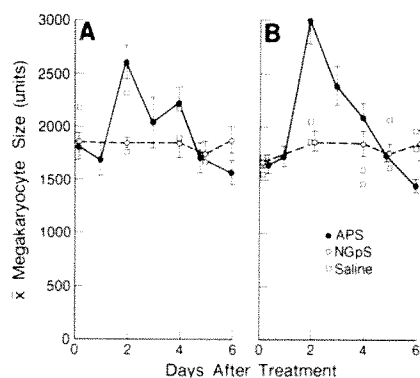


Fig 3. Areas of stage III megakaryocytes of (A) *nu/nu* and (B) *nu/+* mice after a single injection of APS, NGpS, or saline given at time zero. Values for APS and NGpS are the mean $\pm$ 1 SEM for 4 to 12 mice; individual values for saline treated mice are shown. Mean size for both genotypes was significantly ($P < 0.001$) greater after APS than after NGpS on day 2. 1,000 units of size, as plotted here, is equivalent to an unmagnified area of about $660 \mu^2$.

Hematocrits and leukocyte counts showed no differences between mice injected with APS and those injected with NGpS; reticulocyte counts were significantly higher (2.1 ± 0.2 [SEM] ν $1.1 \pm 0.3\%$) on one occasion, day 4 in *nu/+* mice. In the marrow, *nu/+* mice had significantly more megakaryocytes after APS than NGpS on day 2 ($13.4 \pm 2.1 \nu 8.4 \pm 0.8 \times 10^3$ /tibia), and slight hypercellularity was found on day 6 (*nu/nu*: $11.2 \pm 0.6 \nu 9.3 \pm 0.6 \times 10^6$ cells/tibia; *nu/+*: $11.7 \pm 0.8 \nu 9.1 \pm 0.6 \times 10^6$ cells/tibia).

Numbers of splenic Meg-CFC and GM-CFC are illustrated in Fig 4. Both types of colony-forming cells increased in the spleens of *nu/nu* mice after an injection of APS (Fig 4A and 4B), but only Meg-CFC increased after an injection of NGpS (Fig 4A). In contrast, neither type of colony-

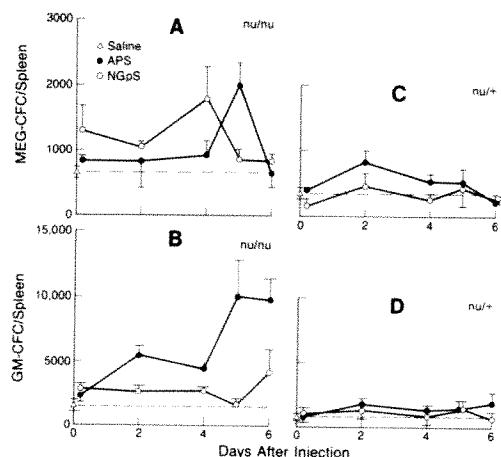


Fig 4. Total splenic Meg-CFC (panels A and C) and GM-CFC (panels B and D) after injection of APS or NGpS; controls were injected with saline. Meg-CFC were significantly ($P < 0.05$) greater than control in *nu/nu* mice (panel A) on day 4 after NGpS and on day 5 after APS. GM-CFC were significantly ($P < 0.05$) greater than control in *nu/nu* mice (panel B) on days 2, 4, 5, and 6 after APS. Other values did not differ from control values. Each point shows the mean and SEM of 3 experiments. The dashed line represents the mean $\pm$ 1 SEM of 10 control mice of the appropriate strain.

forming cell increased in the spleens of *nu/+* mice after injection of APS or NGpS (Fig 4C and 4D). Figure 5 summarizes numbers of bone marrow Meg-CFC, GM-CFC, and BFU-E. None of these cell types increased in the marrow of *nu/nu* mice after injection of APS or NGpS (Fig 5A, 5B, and 5C). Control *nu/+* mice showed an increase in Meg-CFC and GM-CFC four hours after APS (Fig 5D and 5E) and in Meg-CFC four hours after NGpS (Fig 5D); BFU-E did not increase (Fig 5F).

To explore the possibility that the injections of heterologous sera might have recruited latent T cell function in *nu/nu* mice, serial responsiveness of spleen cells to mitogens was determined after the injection of APS or NGpS. The results after APS are presented in Fig 6. As in the untreated mice shown in Fig 1, B cell function, ie, mitogenic response to LPS, was initially intact in *nu/nu* mice, but tended to diminish on days 5 and 6. Incorporation of $^3\text{HTdR}$ into *nu/nu* spleen cells after incubation with PHA or Con A did not exceed the baseline levels observed when cells were incubated in medium alone, except for a weak response to Con A on day 6. Responses to all mitogens were intact throughout the experiment in *nu/+* spleen cells. The results after injection of NGpS are shown in Fig 7. Responses to LPS were intact in both genotypes throughout the experiment as were responses to PHA and Con A in *nu/+* mice. Weak T cell responses were seen in *nu/nu* mice at six days to PHA and Con A and at four hours to Con A.

DISCUSSION

In the present experiments, untreated T cell-deficient *nu/nu* mice were found to be identical to T cell-replete *nu/+* controls with respect to platelet count, megakaryocyte number, and megakaryocyte size. Furthermore, both geno-

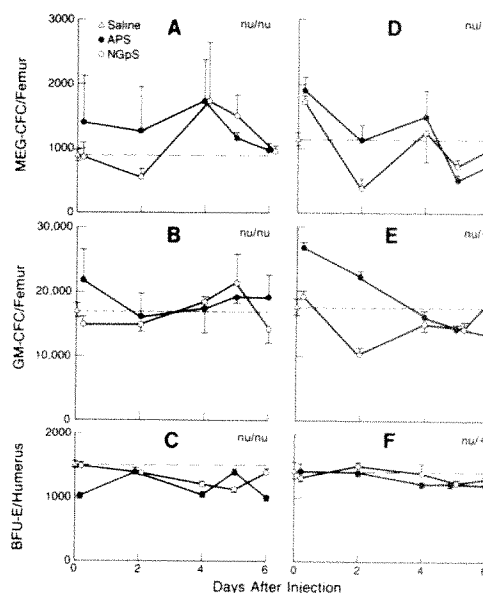


Fig 5. Total femoral Meg-CFC (panels A and D), femoral GM-CFC (panels B and E), and humeral BFU-E (panels C and F) after injection of APS or NGpS; controls for Meg-CFC and GM-CFC received an injection of saline; controls for BFU-E were untreated. The dashed line represents the mean $\pm$ 1 SEM of 10 control mice of the appropriate strain.

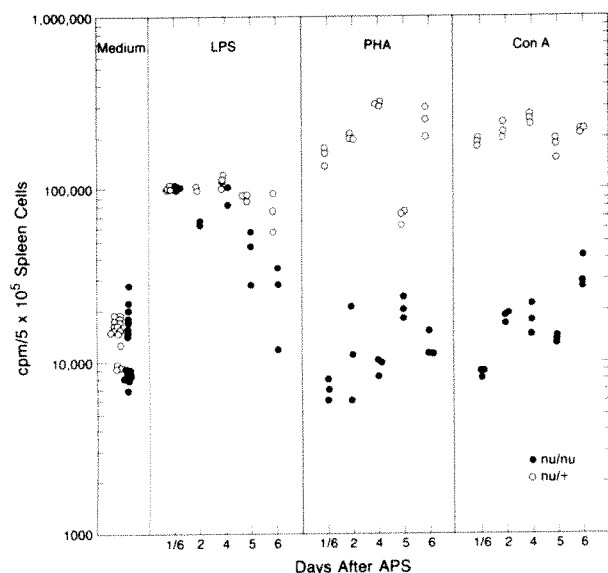


Fig 6. Serial observations of tritiated thymidine incorporation into 5×10^5 spleen cells from nu/nu and nu/+ mice after injection of APS. Assays were performed after incubation in medium alone (background) or in the presence of a mitogen. Each point represents the mean counts per minute (cpm) for quadruplicate cultures. Time on the abscissa refers to the time after injection of APS into the mice that spleen cells were collected and cultured.

types displayed hallmarks of stimulated megakaryocytopoiesis after an injection of APS that have been found in other strains^{4,5,20} and other species^{1-3,21-26}: macromegakaryocytosis and rebound thrombocytosis. Numbers of megakaryocytes increased in nu/+ mice two days after injection of APS, but

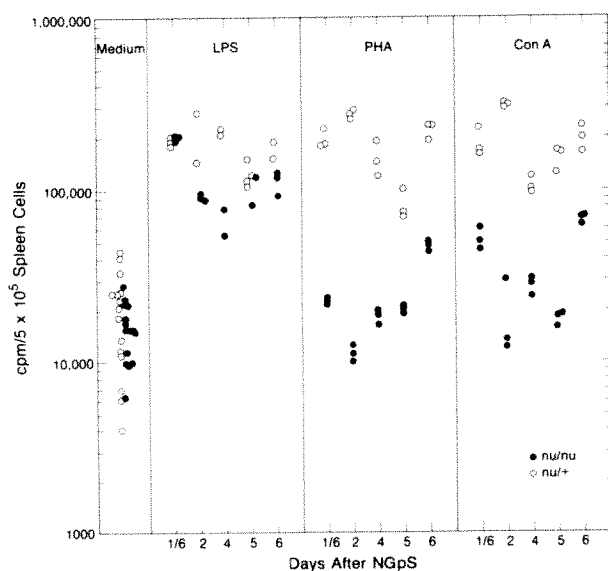


Fig 7. Serial observations of tritiated thymidine incorporation into 5×10^5 spleen cells from nu/nu and nu/+ mice after injection of NGpS. Assays were performed after incubation in medium alone (background) or in the presence of a mitogen. Each point represents the mean counts per minute (cpm) for quadruplicate cultures. Time on the abscissa refers to the time after injection of NGpS into the mice that spleen cells were collected and cultured.

they did not significantly increase in nu/nu mice. Because of variability in the increase in megakaryocyte numbers after a single injection of APS, it is unlikely that this signifies an important difference between nu/nu and nu/+ mice.

Increases in number and size of megakaryocytes, thought to be due to thrombopoietin *in vivo*,²⁷ resemble the effects of megakaryocyte potentiator activity on megakaryocyte colonies *in vitro*.²⁸ It has recently been reported²⁹ that mitogen-activated murine T lymphocytes produce potentiator activity, thus possibly also explaining augmentation of growth of human megakaryocyte colonies by activated T lymphocytes.³⁰ It was suggested that T cells may play an important role in amplifying platelet production in response to inflammation or infection.²⁹ However, the present results demonstrated that a full complement of T cells was not necessary either to maintain normal platelet production or to augment platelet production in response to acute immunothrombocytopenia. Furthermore, the increase of splenic Meg-CFC in nu/nu mice, following administration of NGpS (which did not alter platelet levels), demonstrated that these T cell-deficient mice were capable of responding to an immunological challenge, as has been previously described in C57B1 mice.⁴

As determined by mitogen (PHA and Con A)-induced DNA synthesis, the untreated nu/nu mice used in these experiments had no demonstrable T cells in their spleens. However, others have reported that nu/nu mice are not completely lacking in T cells, but have low numbers of cells that react with antibodies to T cell antigens (reviewed in reference 31). Such cells do not prevent nude mice from accepting grafts of foreign tissue and do not provide the immunological support necessary for a normal life span. Likewise, spleen cells from nu/nu mice, of approximately the same age as those utilized in our experiments, do not appear capable of producing interleukin 2.³² However, it is possible that the small numbers of T cells that are demonstrable with antibodies represent a selected population of T cells that might have hematopoietic regulatory capability. If so, their identification and further study would be of great interest. Macrophages from nu/nu mice have been found to have greater bactericidal activity than those from normal mice.³³ Therefore, it is noteworthy that macrophages have been identified as a potent source of potentiator activity that induces megakaryocyte differentiation *in vitro*.²⁸ Thus, macrophages might have accounted for the normal stimulation of thrombocytopoiesis in nu/nu mice. However, it is also equally possible that neither T cells nor macrophages were involved and that regulation of platelet production is independent of these cell types.

It also has been reported that athymic, but not euthymic, mice have splenic cells capable of suppressing the response of spleen cells to the B cell mitogen LPS.³⁴ If such suppressor cells were activated by the platelet antigen-antibody reaction, the delayed tendency for spleen cell responsiveness to LPS to decline in nu/nu mice after APS but not after NGpS might be explained.

Levels of ³HTdR incorporation that were higher than background were detected on four occasions when nu/nu spleen cells were incubated with the T cell mitogens PHA or

Con A. In all cases the level of incorporation (cpm/ 5×10^5 cells) was considerably less than that found in control spleen cells. However, T cell recruitment, as demonstrated by mitogen responsiveness, did not appear to account for the stimulation of thrombocytopoiesis that was observed in response to immunothrombocytopenia, because only six days after APS injection did nu/nu mice show a weak mitogenic response. By that time, platelet counts were at rebound thrombocytotic levels, and megakaryocyte number and size had returned to normal; that is, stimulation of megakaryocytopoiesis had already run its course.

Similarly, correlations between changes in numbers of colony-forming cells and the state of thrombocytopoiesis in these mice are not obvious. The nu/nu mice showed delayed increases in splenic Meg-CFC after APS or NGpS and in splenic GM-CFC after APS, but thrombocytopenia and stimulation of thrombocytopoiesis occurred only after APS. However, platelet counts on the sixth day after APS were significantly higher in nu/nu than in nu/+ mice. Therefore, it could be proposed that splenic hematopoiesis, as shown by elevated levels of Meg-CFC and GM-CFC in nu/nu but not nu/+ spleens, may have accounted for the difference in platelet levels. Neither Meg-CFC nor GM-CFC increased in the nu/nu bone marrow. The changes in splenic and bone marrow CFC in nu/nu mice resembled those previously described in C57B1 mice,^{4,5} but increases in the spleen were of lesser magnitude and duration. In contrast, nu/+ controls unexpectedly showed no delayed increase in Meg-CFC or GM-CFC in the spleen, but numbers of Meg-CFC were increased in the marrow four hours after APS or NGpS, and numbers of marrow GM-CFC were increased four hours after APS. Since there were no clear-cut differences in thrombocytopoiesis in the two genotypes during the first five days after administration of APS, the influence of these early increases in Meg-CFC and GM-CFC in the marrow on subsequent megakaryocytopoiesis is not clear. These data

suggest that strain differences in CFC responses may exist, and they show that similar thrombocytopoietic alterations can accompany different Meg-CFC and GM-CFC responses to perturbation.

Changes in numbers of CFC could not be attributed directly to thrombocytopenia or to an indirect effect of thrombocytopenia on the marrow, because they also occurred in mice injected with NGpS, which did not produce thrombocytopenia. Similar responses to normal heterologous serum also have been observed in C57B1 mice, and it was postulated that stimulation of the reticuloendothelial system played a role in the response.⁴ In addition, increases in GM-CFC occurred in the marrow of nu/+ mice and the spleens of nu/nu mice only after APS, whereas increases in Meg-CFC followed administration of either APS or NGpS. Others^{5,26} have reported that the proportion of Meg-CFC in DNA synthesis increases in response to thrombocytopenia even if numbers of Meg-CFC do not increase.²⁶ Thus, cell cycle status, which was not measured in our experiments, may be a more sensitive indicator of the involvement of Meg-CFC in thrombocytopoiesis than is their number.

The observations that hematocrits and bone marrow cellularity were lower than normal in nu/nu mice confirms reports of others.^{14,35} Marrow hypocellularity has been reported to be due mainly to low numbers of erythrocytic and lymphocytic cells.¹⁴

In summary, our data indicated that T cell-deficient nu/nu mice regulated thrombocytopoiesis normally both in the unperturbed steady state and in response to acute immunothrombocytopenia. This suggests either that T cells were not required for the regulation of platelet production *in vivo* or that T cell thrombocytopoietic regulatory function resided in the small population of T cells that exists in nu/nu mice. Delayed and nonspecific responses of Meg-CFC suggested that quantitative changes in these cells were not essential for the response to acute immunothrombocytopenia.

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Studies on B Lymphoid Tumors Treated With Monoclonal Anti-idiotype Antibodies: Correlation With Clinical Responses

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Monoclonal anti-idiotype antibodies can be made which are exquisitely specific for B lymphocytic malignancies. We have conducted a clinical trial in which some patients' tumors regressed after infusion of such antibodies. Here, we evaluated characteristics of the antibodies, the tumors, and the patients to determine which features best correlated with the clinical response. Neither the isotype of the murine antibodies, nor their avidity were predictive of clinical outcome. The specific epitope to which the antibodies bound was characterized by immunochemical techniques. Reactivity with a heavy-light chain combinatorial determinant correlated somewhat with clinical effect. Variations in the characteristics of the individual tumors such as antigen sites per cell and ability to modulate the

surface immunoglobulin were not predictive of response. In one patient with prolymphocytic leukemia the anti-idiotype antibody had a direct antiproliferative effect on tumor cells in vitro. This patient's tumor response was explainable by such a direct mechanism. In the other patients, who had lymphomas, therapeutic outcome correlated with the number of host nontumor cells infiltrating the tumor. The vast majority of these nontumor cells were mature T lymphocytes of the Leu 4, Leu 3 (T3, T4) phenotype. Thus, a preexistent host-tumor interaction seems to be important in the in vivo effect of anti-idiotype antibodies in B cell tumors.

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THERAPY WITH MURINE monoclonal antibodies (MoAbs) directed against human tumor cell surface antigens has been tested in several clinical trials.¹⁻¹² Antibodies specific for the idiotype of the surface immunoglobulin (Ig) of B cell malignancies have been among the most successful in inducing regressions of tumor.^{1,3} In our clinical trial, six of ten evaluable patients experienced clinically significant responses of disease during MoAb therapy. One patient's disease regressed completely. Of the five others who experienced a partial response, four had virtually all the tumor which reacted with the antibody eradicated. In those four cases, the original tumor population was replaced by mutant tumor cells which were unreactive with the antibody.^{13,14} However, four other patients treated with anti-idiotype antibodies had no tumor response. It is important to determine which variables pertaining to the antibodies, tumors, or patients may have influenced the clinical outcome. We previously described a correlation between the numbers of normal T cells infiltrating the tumor with response to anti-idiotype antibody therapy.¹⁵ Other variables may also be important. A unique antibody was developed for each patient. Thus, the antibodies potentially differed from each other in isotype, in avidity of binding, and in epitope specificity.

In addition to the properties of the antibodies, differences in the tumors are potentially important. Normal B lymphocytes are responsive to many regulatory substances which promote or inhibit proliferation and/or differentiation.¹⁶⁻²³ Anti-Ig or specific antigen, which, like anti-idiotype antibodies, bind to the surface immunoglobulin, can stimulate or inhibit the proliferation of normal B cells, even in the absence of other mitogenic factors.^{16-18,23,24} Jerne proposed that autologous anti-idiotype antibodies exert a major inhibitory force on B cell clones in the immune response.²⁵ In addition, the maturational state of B cells greatly influences their dependence on growth factors and their receptiveness to immune control, including that mediated by anti-Ig antibodies.^{16,17} B cell tumors appear to represent various stages of normal differentiation, reflected by their morphologic appearance,

ability to secrete immunoglobulin, and tissue localization. By analogy, the differentiation state of a tumor may alter its response to anti-idiotype antibody.

Patients with B cell lymphoma can be immune deficient as a consequence of their disease and/or treatment.^{3,26,27} Anergy, frequent infections, hypogammaglobulinemia, and cytopenias are common problems in patients with B cell malignancies, but vary in severity from patient to patient. These patients might thus have effector systems for antibody-mediated tumor responses. The natural history of low grade B cell lymphoma is characterized by long periods of quiescent disease and occasionally spontaneous regressions.²⁸ Some host antitumor response may be operative in these patients and the anti-idiotype antibody might augment that interaction.

Thus, analysis of the factors important in determining the outcome of anti-idiotype treatment is complicated by the large number of variables. Each patient, his tumor, and his antibody are unique. In this study, we attempted to correlate a variety of in vitro studies of the antibody, tumor, and patient with the clinical response.

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MATERIALS AND METHODS

Patients

Patients in this study and their treatment with anti-idiotypic antibody were described in detail in a prior publication.³ Briefly, all patients had B lymphoid malignancies, eight with nodular lymphoma and one each with large cell lymphoma, prolymphocytic leukemia, and diffuse small cleaved cell lymphoma with foci of large cell lymphoma. Monoclonal anti-idiotypic antibodies were made for each patient's tumor as previously described.²⁹ Tumor samples obtained from the peripheral blood, bone marrow, lymph nodes, skin masses, or spleen of each patient had been frozen in liquid nitrogen and were available for this study either as viable cell suspensions or as frozen tissue fragments.

Immunophenotyping and Enumeration of Subsets

Tumor samples taken prior to therapy were evaluated by routine pathologic analysis, immunoperoxidase staining of frozen sections, and immunofluorescence analysis of single cell suspensions using methods that have been previously described.³⁰⁻³³

Rescue Hybridization

In order to obtain idiotypic protein from the nonsecreting tumor cells, "rescue fusions" were performed with mouse myeloma or heteromyeloma cells as previously described.^{34,35} The resulting hybrids were screened for secretion of human heavy and light chains of the appropriate type by enzyme-linked immunosorbent assay (ELISA). The secreted proteins were purified by immunoaffinity chromatography and used in the assays described below.

Quantitation of Surface IgM

The relative amount per cell of surface immunoglobulin on the different tumors was obtained by flow cytometry using the FACS 440 fluorescence-activated cell sorter (Becton Dickinson, Mountain View, Calif) by methods previously described.³ The mean linear fluorescence intensity³⁶ was determined on single cell suspensions of tumor stained with a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody, 1D12, which is reactive with human μ heavy chain.

Binding constant determination

Equilibrium plate method. Anti-idiotypic antibody was metabolically labelled with ⁷⁵Se-selenomethionine (Amersham Co, Arlington Heights, Ill). Approximately eight million hybridoma cells were incubated for 16 hours in methionine-free RPMI 1640 (GIBCO, Grand Island, NY) containing 5% dialyzed heat-inactivated fetal calf serum. After the labelling period, cells were removed by centrifugation and the supernatant was dialyzed extensively against phosphate buffered 0.9% saline (PBS) to remove unincorporated label. The concentration of anti-idiotypic antibody in the labelled supernatant was determined by immunoassay. The percent of protein-bound radioactivity in the form of active antibody was determined by binding at equilibrium to a vast excess of idiotypic (tumor cells) or goat anti-mouse Ig (coupled to Sepharose beads). The fraction of the total radioactivity that was bindable varied from 24% to 50%.

The binding constant determination was performed in a microtiter plate (Cooke Engineering, Alexandria, VA). Wells were coated with 100 μ L of diluted idiotypic protein or an irrelevant IgM and then incubated with 150 μ L of PBS/bovine serum albumin (BSA) for one hour. After washing, 100 μ L of serially diluted labelled anti-idiotypic was added to the wells. After a six-hour incubation at room temperature, the supernatants were removed and the plates were

quickly washed four times. The dry wells were cut with a hot wire device and counted in a gamma counter. All experimental points were measured in triplicate.

The free counts in each well were determined by subtracting the bound counts from the total input counts. The ratio of bound/free was plotted v bound and a slope of their curve was determined,³⁷ and the binding constant was determined according to the following equation:

$$K = \frac{-(\text{slope})(SA)10}{4.44(\text{mol wt})} \times 1.4(\text{vol})$$

SA = cpm/ μ g
vol = 0.1 mL
mol wt = 150,000

Soluble complex method. The second method used to measure antibody avidity was a modification of the method reported by Griswold and Nelson.³⁸ This method is based upon thermodynamic principles which predict that the binding of antibody to antigen will decrease as the total reaction volume is increased, provided the number of moles of antigen and antibody are held constant. The rate of this decrease is related to antibody avidity.

The assay was performed by mixing anti-idiotypic with a 3-fold excess of idiotypic, and diluting this mixture 1:5 and 1:10 into PBS/10% fetal calf serum. After a four-hour incubation, 50 μ L aliquots of these mixtures were added, in quadruplicate, to wells of a 96-well microtiter plate coated with goat anti-mouse IgG antibody. The concentration of mouse anti-idiotypic initially mixed with idiotypic was previously determined to be nonsaturating on the anti-mouse IgG coated plate. After another four-hour incubation, plates were washed and a horseradish peroxidase (HRP)-coupled goat anti-human IgM antibody was added followed by 2,2'-azino-di-(3-ethylbenzthiazoline) sulfonic acid (ABTS) substrate to detect anti-idiotypic-idiotypic complexes bound to the plate. Avidity was calculated utilizing the BASIC program described by Griswold and Nelson.³⁸ Given the initial idiotypic concentration, the dilution factor, and the fraction idiotypic bound at two different reaction volumes the program determines an avidity value using a binary search algorithm.

Western Blots

Probing of purified proteins with radioactive antibodies was performed following the method of Burnette.³⁹ Idiotypic protein from each patient was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system in both reduced and unreduced states. The protein was electrophoretically transferred to nitrocellulose (0.45 μ m, Millipore, Bedford, MA) overnight. The nitrocellulose was incubated 2 to 8 hours in PBS/5% nonfat dry milk (Carnation, Los Angeles) (PBS/NFDM). First stage antibody at 5 to 10 μ g/mL in PBS/NFDM, was followed by an ¹²⁵Iodine-labelled goat anti-mouse Ig (TAGO, Burlingame, Calif) also in PBS/NFDM. The nitrocellulose was washed between steps with 0.9% saline and 0.9% saline/0.05% NP-40. Autoradiography was performed after the last wash.

Blocking of a Monoclonal Antihuman μ (clone 1D12) with Antiidiotypic Antibody

Further definition of the fine epitope specificity of the anti-idiotypic antibodies was determined by their ability to compete for binding with a murine IgG1 MoAbs specific for human μ heavy chain, to the appropriate idiotypic protein. Tumor cells stained by directly fluoresceinated 1D12 antibody in the presence or absence of anti-idiotypic antibody were analyzed by flow cytometry and their mean fluorescences were compared. Tumor cells frozen in 10% dimethyl sulfoxide (DMSO) were thawed and viable cells were isolated using Ficoll-Hypaque density gradient centrifugation.

Twenty-minute incubations of 10^6 cells with 100 μ L of 10 μ g/mL of first stage antibody diluted in PBS/BSA/0.01% sodium azide were performed at 0°C. The cells were washed twice with cold PBS/BSA/azide. The final antibody, 1D12FITC, was added to the cells which were incubated on ice, washed, and then fixed in 10% paraformaldehyde in PBS. Twenty thousand cells were counted in the flow cytometer and their mean fluorescence in linear units was determined.⁴⁴ The percent blocking was determined using the following equation:

% blocking

$$= 1 - \frac{(\text{mean F anti-id} + 1\text{D12FITC} - \text{background F})}{(\text{mean F irrelevant MoAb} + 1\text{D12FITC} - \text{background F})} \times 100$$

Modulation of Surface Idiotype Immunoglobulin

Aliquots of single cell suspensions of frozen biopsy specimens were thawed and viable mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. In order to induce modulation, aliquots of the tumor cell suspension were incubated with MoAbs in 5% CO₂ at 37°C for varying periods of time. Cultures were started at different times to allow simultaneous staining of cells incubated for 0, 1, and 24 hours. After incubation, the cells were washed extensively with PBS/BSA and then stained for flow cytometry as described above. First stage antibodies used in the staining included an appropriate irrelevant MoAb, the appropriate anti-idiotype MoAb, and 1D12 MoAb. Binding of MoAb to the cells was detected with fluoresceinated goat anti-mouse IgG. The mean linear fluorescence was determined using a FACS IV (Becton Dickinson, Mountain View, Calif).⁴⁰ The percent modulation observed for a particular time and MoAb concentration was determined as follows:

$$\% \text{ modulation} = 1 - \frac{(\text{mean F modulated tumor}) - (\text{mean F tumor} + \text{second step})}{(\text{mean F unmodulated tumor}) - (\text{mean F tumor} + \text{second step})} \times 100$$

Tumor Cell Proliferation Assays

Two hundred thousand viable cells obtained from either a pretherapy tumor biopsy or peripheral blood mononuclear cells, if more than 90% were idiotype positive, were added to a 96-well microtiter plate in 200 μ L of complete medium supplemented with sodium pyruvate (1 mmol/L) and 2-mercaptoethanol (10^{-4} mol/L). One half of the wells also contained 1 μ g/mL phytohemagglutinin (PHA) (Wellcome, Greenville, NC) and phorbol myristate acetate 10 ng/mL (PMA) (Sigma Chemical Co, St. Louis, MO). To triplicates the appropriate anti-idiotype antibody, 1D12 (anti μ) or an isotype matched control antibody were added at a concentration of 10 μ g/mL. The plates were incubated in 5% CO₂ at 37°C for 2, 4, or 6 days and then pulsed with ³H-methylthymidine at a concentration of 1 μ Ci per well. The cells were harvested and counted as previously described.²¹

RESULTS

Murine Antibody Isotype

The isotypes of the MoAbs used to treat the eleven patients are listed in Table 1. Strict criteria were used to define the specificity of each anti-idiotype antibody, and antibodies only of the IgG class were selected. However, no selection was imposed for IgG subclasses. In most instances the first hybridoma clone obtained with the appropriate specificity and stability was chosen for the therapeutic trial. The higher frequency of IgG1 anti-idiotype MoAbs reflects their greater frequency among all the hybridomas generated. Six patients received IgG1, two IgG2a, two IgG2b, and one received three antibodies, one of each isotype. There was no correlation in this study between the IgG subclass of the antibody and the occurrence of a tumor response. The only patient to achieve complete elimination of tumor received an IgG2b antibody; the next most dramatic responder was treated with an IgG2a. However, four of six patients infused with IgG1 anti-idiotype MoAb experienced objective

Table 1. Immunohistochemistry

Patient	PK	FS	BL	RD	BJ	CJ	CP	CG	TG	KL	PE
Treatment order	01	02	03	04	05	06	07	08	09	10	11
Response*	100	90	0	60	10	55	0	45	0	NE	90
Idiotype											
Phenotype	$\mu\lambda$	$\mu\kappa$	$\mu\kappa$	$\mu\lambda$	$\mu\kappa$	$\mu\kappa$	$\mu\lambda$	$\mu\kappa$	$\mu\kappa$	$\mu\lambda$	$\mu\kappa$
Density†	219	1479	389	224	ND	370	790	ND	186	ND	ND
Antibody	γ 2b	γ 1	γ 1	γ 1	γ 2b	γ 1	γ 1	γ 1	γ 2a	γ 1, γ 2a, γ 2b	γ 2a
Epitope											
1D12 Block‡	15	77	9	62	ND	69	15	ND	81	10, 62, 72	ND
Chain§	HL	HL	H	HL	H	H	—¶	ND	HL	ND	HL
Affinity											
Rank	4	5	2	8		6	7		3		1
Plate#	230	64	>500	ND	ND	48	45	ND	410	ND	ND
Dilution#	100	41	530	<1	ND	34	29	ND	ND	38, 220, 36	1000

Abbreviations: ND, not determined; NE, not evaluable.

*Percent reduction in overall measurable tumor.

†Estimate of surface immunoglobulin per cell expressed as mean linear fluorescence of 2×10^5 tumor cells stained with 1D12-FITC.

‡Expressed as percent of maximal blocking of 1D12 by itself.

§Reactivity of anti-idiotype antibody with idiotype protein on Western blot; H, heavy chain; HL, heavy and light chain together.

||SDS-sensitive idiotype, renaturable.

¶SDS-sensitive idiotype, not renaturable.

#Binding constant $\times 10^7$.

responses. Two other evaluable patients treated with IgG2 antibodies had no response.

Idiotypic Protein

All tumors described in this study expressed IgM on their cell surface. Four expressed λ light chain and seven expressed κ light chain. Six patients (one λ , five κ) secreted IgM which could be detected in the serum in concentrations of 0.1 to 400 $\mu\text{g/mL}$.³ This secreted idiotypic protein was an IgM pentamer as determined by gel electrophoresis of samples isolated from the serum of two patients and from supernatant from "rescue hybrids" of the tumors of three others³⁴ (unpublished data). Although the presence of free idiotype protein in the serum could block binding of anti-idiotype to cellular idiotype, it did not preclude a favorable therapeutic result if a dose of anti-idiotype MoAb sufficient to achieve extravascular penetration was infused.

Estimation of the amount of surface IgM per tumor cell, revealed a sevenfold difference between the highest and lowest of the tumors tested (Table 1). The tumor cells from patient FS, with prolymphocytic leukemia, had nearly twice as much surface Ig as the next highest expressing tumor. Cells of the majority of patients clustered in the lower range, and there was no correlation between surface Ig expression and clinical response.

Epitope

The surface IgM of each individual tumor has a unique variable region with its own idiotypic determinants, but the majority of the molecule is made up of constant protein sequences shared by them all. The anti-idiotype MoAbs were by definition specific for determinants dependent upon the variable regions of the heavy and/or light chains of the tumor specific immunoglobulin. This epitope could be further defined by several methods. A particular MoAb, 1D12, which is specific for the human μ heavy chain and presumably reactive with a determinant in the constant region proximate to the variable region, could be significantly blocked by six of the ten anti-idiotype antibodies that were tested (Table 1). It is unclear whether the anti-idiotype antibodies that block sterically hinder the binding of 1D12 or if their binding produces changes in the tertiary structure which alter the 1D12 binding site. Different anti-idiotype antibodies against the same IgM molecule can differ in their effects on 1D12 antibody binding presumably because they bind to different sites (idiotypes) (Fig 1).

Immunoblots of reduced and unreduced idiotype protein provided additional information about the idiotypic determinant recognized by each antibody (Table 1). This technique can distinguish between antibodies which bind to an idiotope located on the isolated heavy or light chain, separated by gel electrophoresis after reduction, from those that require the intact immunoglobulin. Examples of both situations are displayed in Fig 2. In two cases, the electrophoresis procedure produced changes in the conformation of the idiotype of the unreduced molecule which prevented binding by the antiidiotype antibody.⁴¹ The sodium dodecyl sulfate used in the electrophoresis was believed to be the factor

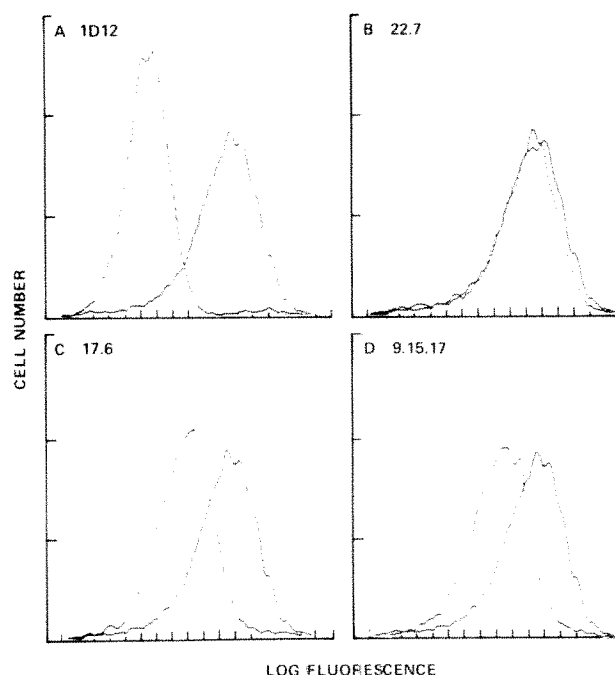


Fig 1. Anti-idiotype antibody blocking of 1D12. Histograms show the logarithmic fluorescence intensity after staining of a cell line derived from the tumor of patient KL which expressed idiotype-positive surface IgM. The cells in each histogram were stained with 1D12 anti- μ MoAb conjugated to fluorescein isothiocyanate (1D12-FITC) as a second step. The solid line in each panel represents initial incubation of the cells with a nonbinding isotype control antibody, showing maximal staining with the 1D12-FITC. The dashed line represents cells incubated previously with unconjugated antibody, (A) 1D12, (B) 22.7 anti-idiotype, (C) 17.6 anti-idiotype, and (D) 9.15.17 anti-idiotype. Displacement of the curve to the left represents the relative ability of the first step antibody to block the binding of 1D12-FITC to its epitope.

responsible and after washing with urea and NP-40 the binding site was restored in one of these two cases.⁴² Of eight idiotype proteins evaluable by this method, five reacted only with the assembled heavy-light chain molecule and three reacted with both the isolated heavy chain and the intact antibody. None of the anti-idiotype MoAbs reacted with the free light chain.

The ability to block 1D12 and the chain reactivity on Western blot analysis did not correlate with each other and appear to evaluate different facets of idiotype fine specificity. Blocking of the 1D12 binding site did not correlate with clinical outcome. However, the data do suggest that reactivity with an HL combinatorial determinant may be important, that is, 4/5 responders *v* 1/2 nonresponders had HL reactivity.

Binding Constants

Another potentially important feature of the anti-idiotype antibodies is their affinity or avidity. Determination of affinity requires accurate measurements of the concentrations of free and antibody-bound antigen under various conditions. In this case, both antigen and antibody are large proteins and complexes cannot be easily separated from free

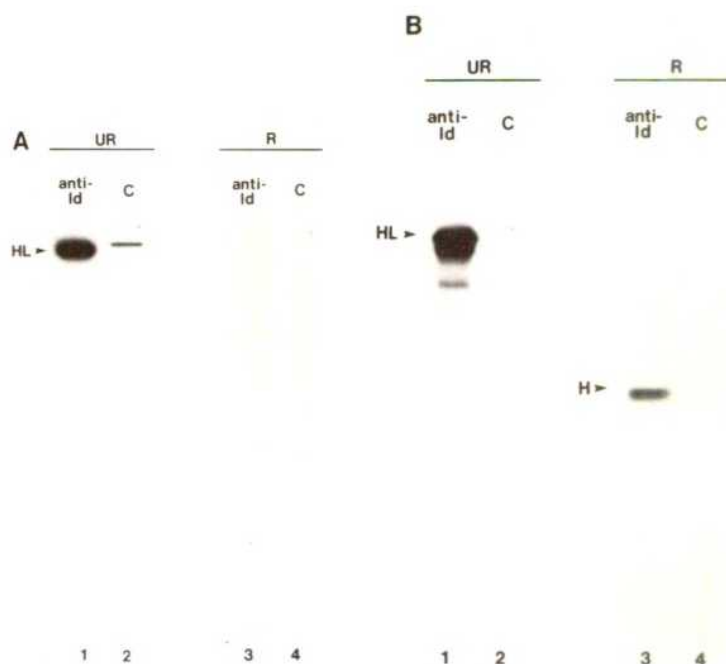


Fig 2. Immunoblotting of idiotype proteins with anti-idiotype antibodies. Idiotype protein was subjected to SDS-PAGE under reducing lanes 1 and 2 (R) and nonreducing lanes 3 and 4 (UR) conditions and electroblotted onto nitrocellulose membranes. Purified idiotype from patient PK (panel A) and CJ (panel B) were probed with the appropriate anti-idiotype (anti-id) or isotype matched control (C) antibodies and binding was detected with ^{125}I -goat anti-mouse Ig. Anti-idiotype for patient PK reacted with only the combined heavy and light chain and not with the isolated heavy or light chain. Anti-idiotype for patient CJ reacted with both isolated heavy chain and the intact molecule.

antigen. Two relatively independent methods were used to determine the binding constants of the anti-idiotypic MoAbs for which purified idiotype was available (Table 1). Both methods partition unbound antibody from bound antibody by fixing the immune complexes to a solid phase. In the equilibrium plate method the concentrations of bound and free anti-idiotype antibody are determined after interacting with the idiotype which was initially fixed to a plastic plate. The soluble complex method measures the amount of soluble complex formed by a fixed ratio of antibody and antigen at different total protein concentrations. These independent methods are clearly in agreement regarding the rank order of avidity of the MoAbs studied and remarkably close in their absolute values. A thousandfold difference existed between the most avid (PE) and the least avid (RD) antibodies. The highest and lowest values, however, were outside the most accurate quantitative range of both assays and the numerical values assigned to these two cases are less precise than the others. The rank order of these antibodies, however, is clear, and there was no correlation between this feature of the antibody and clinical response.

Modulation

Surface immunoglobulin, like many cell surface proteins, can move within the plane of the membrane and when cross-linked by multivalent antibody may be internalized causing the disappearance (modulation) of the surface antigen. The anti-idiotypic MoAbs caused a variable degree of modulation of cell surface idiotypic immunoglobulin both in vitro and in vivo. Fig 3 demonstrates differences in the modulability by their respective anti-idiotypic antibody of the tumors tested. The time dependence of modulation can be appreciated, as all tumors demonstrate increased modulation between one and 24 hours of incubation. Considerable variation existed among the tumors as to the maximal modulation which could be induced. CP exhibited no modulation at the $1\text{ }\mu\text{g/mL}$ concentration of anti-idiotypic MoAb and significantly less than the others at the higher concentration. Others were able to modulate 50% to 90% over 24 hours. Tumor cells of patient CG underwrote modulation in vivo as demonstrated in a lymph node specimen removed during treatment with anti-idiotypic MoAb (data

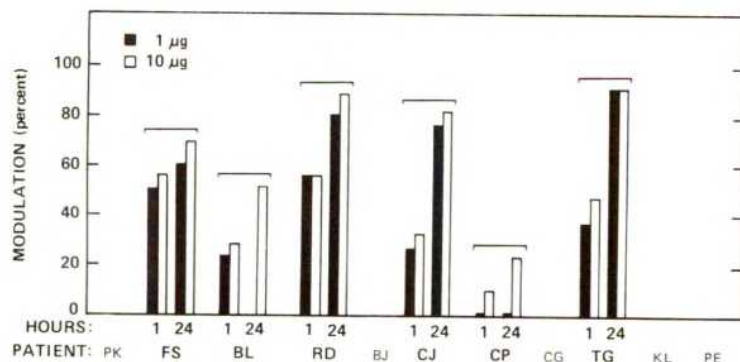


Fig 3. Modulation of surface immunoglobulin by anti-idiotype antibody. The tumor cells evaluated in Fig 3 were incubated for varying times with 1 or $10\text{ }\mu\text{g/mL}$ of anti-idiotypic antibody. After staining with FITC-labeled goat anti-mouse Ig, the mean fluorescence of the cells was determined and related to that for cells incubated with a nonbinding control. Complete disappearance of surface immunoglobulin is equivalent to 100% modulation. The analysis was not performed on patients PK, BJ, CG, KL, and PE.

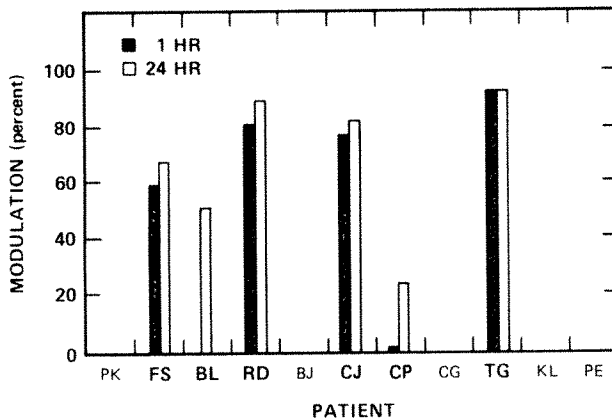


Fig 4. Modulation of surface immunoglobulin by 1D12 antibody. The patient's tumor cells were incubated for varying times with 1D12 (anti- μ chain MoAb) at a concentration of 10 μ g/mL. The percent modulation was determined as described in Fig 3.

not shown). Modulation appeared to be a function of both the biologic behavior of the tumor cell and the MoAb used. Modulation induced by the anti- μ MoAb 1D12 (Fig 4) was similar to that induced by the anti-idiotypic MoAb, however, the rate at which it occurred and the absolute degree of surface antigen decrement varied. Avidity, epitopic specificity, and clinical response did not correlate with in vitro modulation.

Effects of MoAb on In Vitro Proliferation of Tumor

In order to investigate whether the anti-idiotypic MoAbs had a direct effect upon their respective target tumor cells, we cultured the cells in vitro with the anti-idiotypic or with the anti- μ 1D12 antibodies in the presence or absence of mitogens PHA and PMA for up to seven days. The results

are displayed in Table 2. The addition of the mitogens PMA and PHA increased the baseline proliferation of the cells by a factor of three to ten (Table 2A), but the effects of the antibodies were the same whether or not the mitogens were present (Table 2B). The cells incubated without antibody maintained a stable proliferative state over the seven-day period. Three of the tumors were unaffected by the addition of either the anti-idiotypic or the anti- μ antibody. Two other tumors were mildly affected (TG was suppressed; BL was stimulated) by both their respective anti-idiotypic antibody and by anti- μ . Neither of these patients experienced any change in their tumors upon treatment with their anti-idiotypic antibodies. By contrast, tumor cells from patient FS were markedly suppressed by both anti-idiotypic and anti- μ MoAb. This patient, with prolymphocytic leukemia, had a dramatic and sudden response to anti-idiotypic antibody therapy, in contrast to the rather gradual responses observed in other responding patients.³ The rapid tempo of the clinical response in patient FS and the unusual susceptibility of his tumor cells to a direct antiproliferative effect by anti-Ig antibodies in vitro suggest the mechanism for his response was different from that for the other patients.

Host Factors

The various tissues obtained from the patients were analyzed by immunofluorescence and flow cytometry of cells in suspension and by immunoperoxidase staining of frozen tissue sections. All specimens contained numerous infiltrating normal cells. As previously reported,¹⁵ the normal host cells infiltrating the tumor were composed primarily of T lymphocytes. These T cells were predominantly of the Leu 3, Leu 4 (T4, T3) positive (helper) phenotype (Table 3). Smaller numbers of Leu 2, Leu 4 (T8, T3) positive (cytotoxic, suppressor) T cells were also present, as were a small,

Table 2. Proliferation Assays

	PK	FS	BL	RD	BJ	CJ	CP	CG	TG	KL	PE
A. CONTROL MoAb*											
Day 3	ND	145	5.5	20	ND	2	21	ND	32	ND	ND
— Mit†	ND	95	0.7	3	ND	0.8	4	ND	4	ND	ND
Day 5	ND	55	7	26	ND	2.5	13	ND	31	ND	ND
— Mit†	ND	10	0.5	2.5	ND	1	5	ND	3	ND	ND
Day 7	ND	50	10	29	ND	3	12	ND	30	ND	ND
— Mit†	ND	4	0.5	2.5	ND	1	3	ND	3	ND	ND
B. ANTIIDOTYPE MoAb‡											
Day 3	ND	79	145	100	ND	100	100	ND	74	ND	ND
Day 5	ND	63	157	100	ND	100	100	ND	73	ND	ND
Day 7	ND	14	149	100	ND	100	100	ND	74	ND	ND
ANTI-MoAb‡											
Day 3	ND	62	120	100	ND	100	100	ND	75	ND	ND
Day 5	ND	45	125	100	ND	100	100	ND	75	ND	ND
Day 7	ND	14	112	100	ND	100	100	ND	76	ND	ND

ND, not determined.

*Proliferation of tumor cells as measured by ³H-thymidine incorporation in counts per minute $\times 10^{-3}$.

†Wells without mitogens.

‡Reported as percent of the ³H-thymidine incorporation seen with control MoAb (Table 2A)

Table 3. Pretherapy Biopsy Specimens Analysis with Monoclonal Antibodies*

Patient	Total Pre-Therapy Biopsies†	Diagnosis‡	Tissue§	Leu-4	Leu-3	Leu-2	Leu-7	Tumor Cells	Response
PK	2	N/DLPD	LN	51	36	7	7	30	100
FS	1	PLL	BM	8	11	1	0.5	83	90
BL	1	N/DLPD	LN	15	13	6	2	79	0
RD	1	DLPD/LCL	LN	31	14	8	2	80	60
BJ	1	NML	LN	16	15	5	2	80	10
CJ	2	NLPD	LN	34	20	8	4	88	55
CP	2	NLPD	LN	19	16	4	2	86	0
CG	2	NML	Spleen	23	24	3	2.7	79	45
TG	1	NLPD	Spleen	7	7	1	0.4	90	0
KL	2	DLCL	LN	6.6	10	1.7	ND	80.7	NE
PE	3	N/DPLD	LN	77	70	9	5.1	26.1	90

Abbreviations: ND, not determined; NE, not evaluable.

*Reported as percent of total cells positive for each antibody as determined by flow cytometry, except for patient PK and all Leu 7 data which was determined from immunoperoxidase-stained frozen sections.

†Numbers reported are representative of all pretherapy biopsies.

‡NLPD: nodular lymphocytic poorly differentiated, DLPD: diffuse lymphocytic poorly differentiated, PLL: prolymphocytic leukemia, LCL: large cell lymphoma, NML: nodular mixed lymphoma.

§LN: lymph node, BM: bone marrow.

||Percent reduction in overall measurable tumor.

variable number of Leu 7 positive (NK/K) cells. Leu 7 positive cells were large and localized to follicles in patients with follicular histology. In patients with follicular diseases the host infiltrate cells were localized predominantly in the interfollicular areas.

Interestingly, we observed that ratios of malignant B cells to nontumor T cells existed which were characteristic for each patient. For instance, in one patient, pretherapy specimens were obtained from three different tissue sites (lymph node, skin, and bone marrow) at two separate time points. All three biopsies were found to have maintained a similar ratio of tumor cells to host infiltrate (Fig 5).

There was a correlation between the number of nontumor T cells present in biopsy specimens prior to anti-idiotypic therapy and the response to that therapy (Fig 6). This correlation was especially striking in view of the failure of the many other parameters of the therapeutic MoAb, the tumor cell, and the host mentioned above to predict response to anti-idiotypic therapy. Of the two patients with tumors containing 56% to 80% Leu 4 positive cells in the biopsy, the disease in one completely remitted with anti-idiotypic MoAb and that in the other showed an impressive response. The difference in the proportion of T cells in these two patients compared to the other patients was statistically significant ($P < .001$). The patients with lesser responses had between 23% and 34% Leu 4+ cells in their pre-MoAb therapy tissue biopsies. The patients who had no tumor response all had less than 16% T cells in their specimens. This evaluation excludes patient FS, whose disease was a leukemia rather than a lymphoma and whose tumor response appeared to be mediated by a direct antiproliferative effect.

DISCUSSION

A series of patients with B cell malignancy has been treated with MoAbs directed against an idiotypic determinant of the immunoglobulin molecule present on the

surface of their tumor cells. A spectrum of clinical outcomes has been observed, ranging from complete and durable remission to no effect.^{1,3} Some of the factors that influence the therapeutic outcome have been previously identified and include the presence in the serum of idiotype protein, the occurrence of an immune response against the foreign MoAb, and the existence within the tumor population of cells with mutations of the idiotype.^{3,13,14} In view of these obstacles, it is remarkable that clinically significant tumor regressions have occurred in over half of the patients who have been studied. In fact, the emergence of tumor cells with idiotype mutations during therapy with an anti-idiotypic antibody implies that the antibody exerted a strong selective force against the idiotype-positive tumor cell population in these cases.

In the current report we have presented a series of studies to elucidate the mechanism of the antitumor effect of the anti-idiotypic antibodies. Since each patient, tumor and antibody were unique and only limited amounts of pathologic material were available from some of the patients, exhaustive studies were difficult to perform. Nevertheless, we attempted to examine the relative roles of the antibody—its class, affinity, and epitope; the tumor—its responsiveness to direct effects of anti-idiotypic antibody and the surface density and modulability of its Ig molecule; and the host—the nature and composition of the cellular infiltrate within the tumor tissue.

The known mechanisms of in vitro antibody mediated cytotoxicity include complement fixation and antibody-dependent cell-mediated cytotoxicity (ADCC). Both of these functions are highly dependent on antibody class as well as the availability of the effector molecules and/or cells. Kipps et al have recently documented the superiority of murine IgG2a over IgG2b and IgG1 antibodies in performing ADCC in vitro with human effector cells,⁴³ and numerous studies in animal models have likewise suggested a superiority of IgG2a antibodies for in vivo antitumor effects.⁴⁴⁻⁴⁶

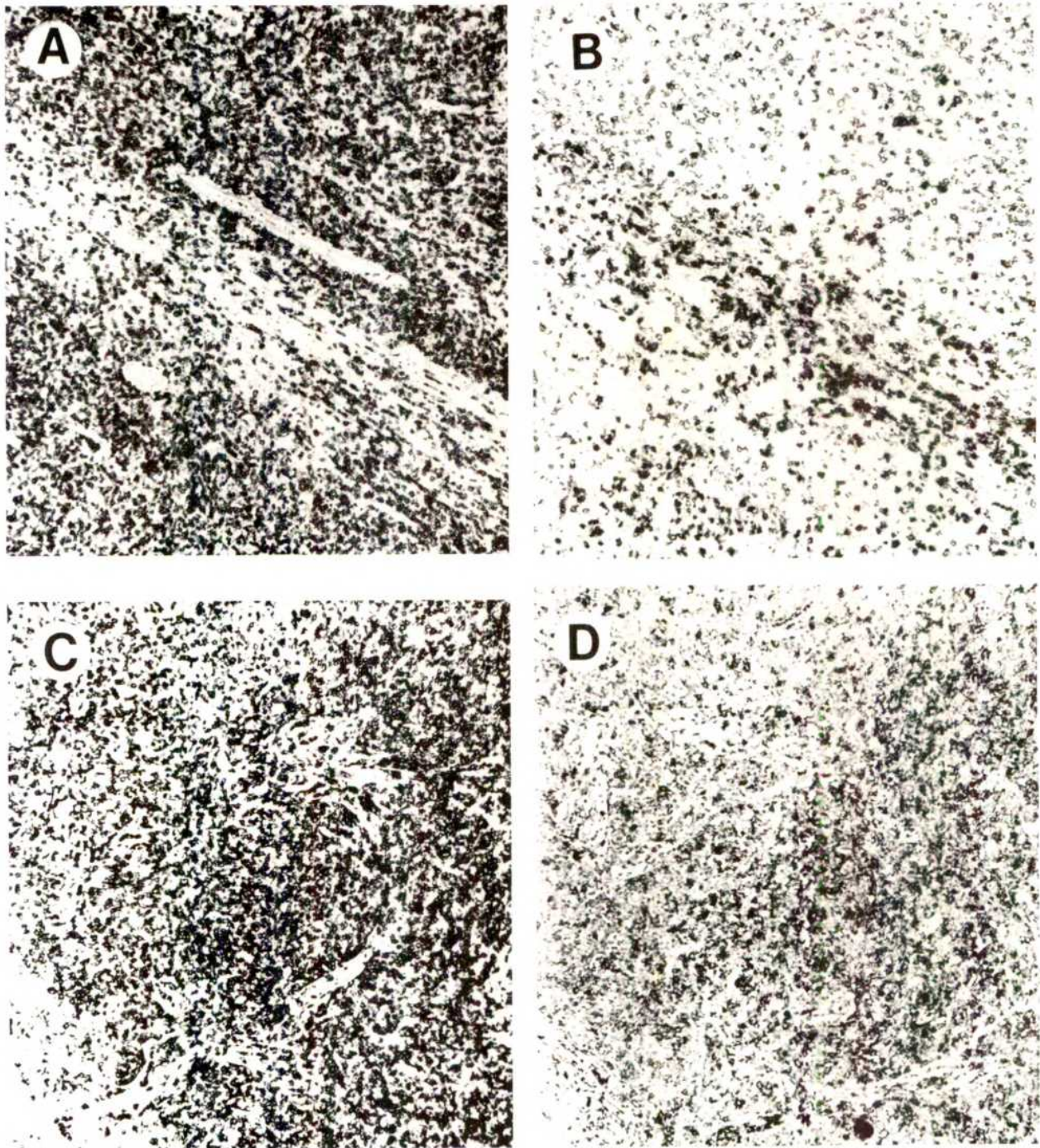


Fig 5. Relative numbers of infiltrating host T Lymphocytes and malignant B cells are demonstrated by immunoperoxidase staining of frozen sections of pretherapy tumor specimens from patient PE. Simultaneous biopsy of lymph node (A,B) and subcutaneous mass (C,D) were performed in April 1982. A bone marrow biopsy (E,F) was performed in August 1984 immediately prior to treatment with anti-idiotype antibody. Frozen sections of each biopsy specimen were incubated with anti-Leu 4, which binds to mature T lymphocytes, (A,C,E), or an antibody specific for a B lymphoid cell surface marker (B-anti- μ , D-anti-B1, F-anti- μ). Cells binding antibody were detected with an immunoperoxidase method and the specimen was counterstained with methylene blue. The large excess of host T lymphocytes to tumor cells is maintained over time and in various tissues involved with tumor.

There was no clear relationship in our clinical trial between antibody class and outcome. Although the most dramatic responses occurred with antibodies of the IgG2 class (one IgG2a and one IgG2b), responses occurred with antibodies of the IgG1 class as well. In addition, we have not observed a

correlation between changes in serum complement levels and clinical outcome. The observations tend to argue against an important role for a direct cytotoxic effect of anti-idiotype antibodies, although they do not rule out such a mechanism.

Whatever the mechanism of antibody effect, it seemed

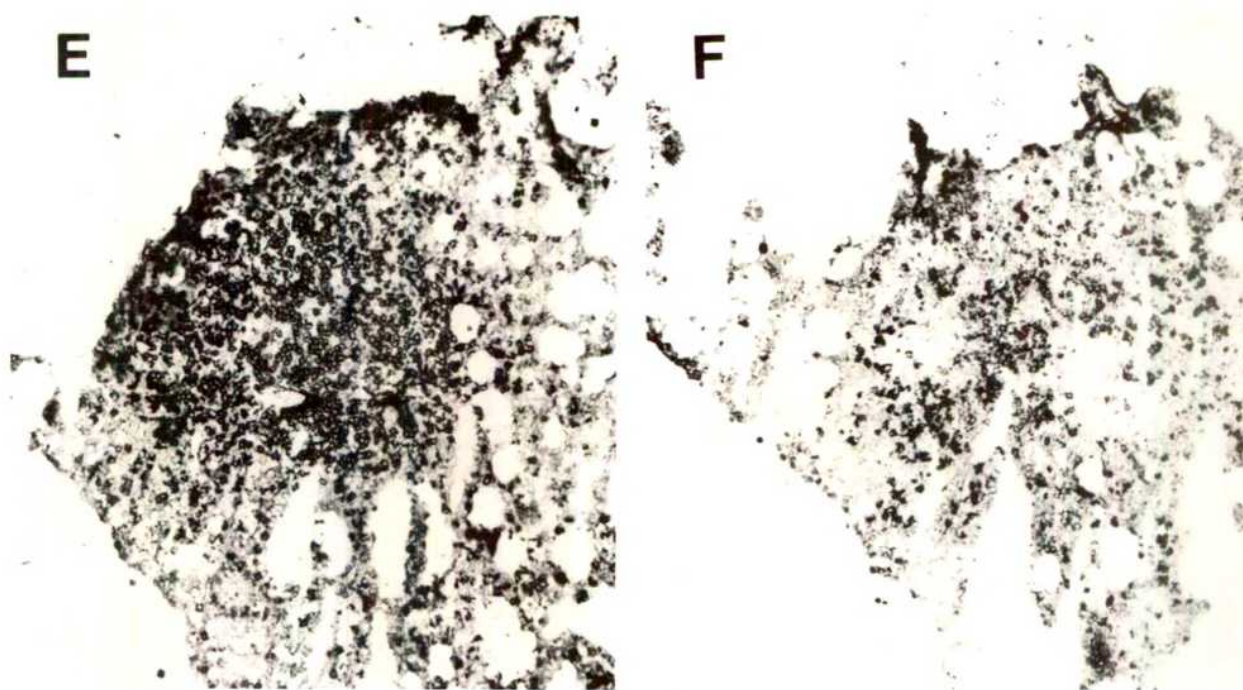


Fig 5. (Cont'd).

likely that the avidity of interaction between antibody and idiotype target should be important. Two different methods were used in this study to estimate antibody avidity. The results were in close agreement, and certainly there was concordance in the rank order of the different antibody-idiotype combinations. Over the range of avidity covered by these particular antibodies no correlation existed with clinical outcome. Apparently the methods that were used to select MoAbs resulted in antibodies of sufficiently high avidity to produce *in vivo* effects.

Although idiotypic determinants are located somewhere within the variable regions of the immunoglobulin heavy and light chains, different anti-idiotypic antibodies can recognize different parts of the variable region structure. For instance, in cases of myeloma proteins which bind known antigens, some anti-idiotypic antibodies inhibit antigen binding and some do not.⁴⁷⁻⁴⁹ If anti-idiotypic antibodies exert a direct regulatory effect on the B cell which bears the relevant target molecule on its surface, the exact portion of the V region to which they bind may be of critical importance in signal transduction. Since in B cell lymphomas no antigen is known to bind to the cell surface idiotype, we used two alternative approaches to investigate the idiotypic determinants. A MoAb reactive with the μ heavy chain was blocked from binding to its respective epitope by certain anti-idiotypic antibodies. Evidently, this anti- μ antibody reacted with a site in the C μ region near the V-C junction. We could then infer that some of our anti-idiotypic antibodies reacted with a site nearby. Some idiotypic determinants are created by a combination of heavy and light chain structures, and others are dependent only upon one of the chains. We tested the antibodies which were used in the therapeutic trials for their ability to react with isolated chains and found that several of

them reacted with determinants on the heavy chains. Steric hindrance of an anti- μ chain antibody showed no correlation with outcome whereas reactivity with an HL combinatorial determinant did show some correlation with clinical effectiveness *in vivo*.

Direct effects of anti-idiotypic or anti-Ig antibodies on normal and neoplastic B cells have been demonstrated in a number of *in vitro* systems.^{16-18,23,24,50,51} These effects have included stimulation of proliferation, inhibition of proliferation, and induction of receptors for BCGF and IL2. The differences seem to depend upon the *in vitro* system, the particular anti-Ig reagent and the state of differentiation of

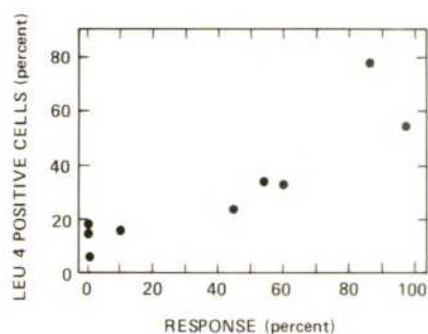


Fig 6. Impact of T cells on response to anti-idiotypic therapy. The percent of Leu 4 or Leu 1 positive cells, as determined by flow cytometry in the pretherapy biopsy of patients is plotted on the vertical axis and the percent response measured by lymphangiogram or computerized axial tomography is plotted on the horizontal axis. The percent of T cells is the average measured in all 15 pretherapy biopsies of the nine patients eligible for this analysis. The two patients with the best responses were biopsied two and three times prior to anti-idiotypic therapy.

the B cell target. The antiproliferative effect of anti-Ig on normal B cells can be prevented by prior treatment with mitogens.¹⁷ It was thus important to investigate the direct effects of the anti-idiotypic antibodies which were used for therapy on their respective tumor target cells. We found that the antibody used in one patient (FS) had a profound inhibitory effect on in vitro tumor cell proliferation. The antibody used in patient BL had a slight stimulatory effect, and that used in patient TG had a mild inhibitory effect on tumor cell proliferation. Those used on the other three patients tested had no measurable effect in vitro. The effects of antibody on the cells were unchanged by concomitant mitogen stimulation. The anti-idiotypic antibodies used for patients BL and TG had no in vivo effect on their tumors, either positive or negative. The kinetics of the tumor response in patient FS were consistent with a direct antiproliferative effect, since there was an acute reduction of tumor cells in the blood and bone marrow. Patient FS was different from the other patients in our series in that he had prolymphocytic leukemia, whereas the others had lymphomas. The FS tumor cells were larger and expressed markedly more surface Ig than the others. Gordon et al found EBV-transformed lymphoblastoid B cells to be inhibited by anti-Ig antibodies.¹⁸ Prolymphocytic leukemia cells might be "fixed" in a normal differentiation state susceptible to regulation by anti-idiotypic antibody.

Modulation, or "capping," of the surface antigen target by MoAb had important implications in systems in which ADCC or antiproliferative effects of antibodies were studied.^{16,23,52} Univalent (nonmodulating) antibodies have been shown to be more cytolytic both in vivo and in vitro than their bivalent counterparts which can cross-link surface antigen and produce modulation.⁵² Capping of surface Ig is required for signal transduction in the antiproliferative and toleragenic effects produced by anti-Ig antibody on neonatal murine B cells.¹⁶ Differences in the capacity for modulation were observed among the patients' tumor cells in this study which might have influenced response to therapy. However, Schroff et al demonstrated the enhancement of modulation by Fc receptor-bearing monocytes.⁵³ The in vitro modulation of the individual tumors observed in this study may have been influenced by the presence of variable numbers of macrophages/monocytes in each suspension. Similarly, the availability of Fc receptor-bearing cells during in vivo therapy might change the tumors' ability to modulate in the presence of anti-idiotypic antibody. Within these limitations, no correlation existed between in vitro modulability and clinical outcome.

In some MoAb clinical trials, the suggestion has been made that an immune response by the patients against the murine immunoglobulin was beneficial.⁵⁴ Theories have been advanced about second and third order immune responses involving idiotypic—anti-idiotypic networks, ultimately leading to an immune attack by the host against the tumor.⁵⁴ In our trial, an immune response against the murine immunoglobulin was documented in several of the patients, and in each case a portion of the response was indeed directed against the idiotypic determinant of the mouse monoclonal antibody.³ However, no support can be found in our data for

a role of the anti-mouse immunoglobulin immune response in the antitumor effect. The two most dramatic tumor regressions occurred in patients who did not make an immune response. Furthermore, in those patients who did make an immune response, no additional tumor regression was ever noted after that point in time.

Although an immune response by the host against the mouse MoAb seems unlikely to be therapeutic, an immune response against the tumor is a possible mechanism. However, this immune response seems to exist in the host prior to the introduction of the MoAb.^{14,15,55-57} In our study, there was a good correlation between clinical outcome and the number of T cells present in the pretherapy tumor tissue. In the two best responding cases the T cells actually outnumbered the tumor cells. Analysis of the cellular infiltrate showed the majority of the T cells to be of the helper/inducer (Leu 3, OKT4) phenotype in each case. Also present in this cell population were Leu 7 positive (putative NK) cells. One view would then be that the MoAb amplified or augmented an ongoing cell-mediated cytotoxic response by the host against the tumor, tipping the balance back in favor of the host. The fact that the T cells were of the Leu 3 phenotype is not inconsistent with this hypothesis, since Leu 3 positive, T killer cells that are restricted in their target cell recognition by the class II MHC structures are well described.⁵⁸ An alternative view is that the interaction between host T cells and tumor cells is a positive or trophic one. Activated T cells are a rich source of growth stimulatory factors for B cells. The fact that T cells were found associated with the tumor in relatively fixed proportions in various sites throughout the body is equally consistent with a positive as well as a negative interaction. In this view, the MoAb would be interrupting or somehow interfering with the trophic effect of the T cells on the growth of the tumor. Whether either of these mechanisms of anti-idiotypic antibody effect are operative can only be the subject of speculation at the present time. Clearly, more observations must be made as more patients are treated. Also, it will be extremely important to isolate the T cells from the tumor specimens, to measure their effects on the tumor cells, and to determine whether anti-idiotypic antibodies alter these effects.

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Polymorphic Glycoprotein-1 on Mouse Platelets: Possible Role of Pgp-1 and LFA-1 in Antibody-Dependent Platelet Cytotoxicity Involving Complement

By Peter J. McCaffery, An Seng Tan, and Michael V. Berridge

The presence of the Pgp-1 glycoprotein on mouse platelets is demonstrated by antibody-binding techniques, by immunoprecipitation, and by transblotting using the monoclonal antibody (MoAb) C71/26 against Pgp-1. C71/26 immunoprecipitates as a broad band of mol wt 87,000 to 100,000 as determined by radioiodination of the platelet cell surface and by the ³H-sodium borohydride labeling technique. Immunoblotting showed Pgp-1 expression on platelets to be quantitatively similar to its presence on macrophages and resolved platelet Pgp-1 into two bands of mol wt 87,000 and 97,000 whereas Pgp-1 on parasite-elicited peritoneal macrophages showed 82,000 and 87,000 mol wt species. Platelets and monocyte/macrophage cells from either peripheral blood or from the peritoneal cavity showed homogeneous binding of Pgp-1 antibody to greater than 97% of cells by flow cytometry. In contrast, lympho-

cytes from peripheral blood or from the spleen showed a heterogeneous binding pattern with 20% to 30% of cells being negative, and the majority weakly positive. In functional studies, MoAbs against CR1 and CR3 substantially inhibited platelet immune adherence, whereas C71/26 showed only marginal inhibition. In contrast, C71/26 and other MoAbs against Pgp-1 inhibited platelet-dependent cytotoxicity of antibody-coated sheep erythrocytes in the presence of C5-deficient mouse plasma whereas M1/70 against CR3 showed no effect. In this assay, MoAbs against the α - and β -subunits of leukocyte functional molecule LFA-1 also inhibited platelet cytotoxicity. These results show that the platelet cell surface moieties Pgp-1 and LFA-1 are involved in or closely associated with antibody-dependent cellular cytotoxicity by platelets.

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Platelets are the second most abundant cell type in circulating blood where their primary function is hemostasis. Another well-established function for platelets is immune adherence to microorganisms through complement receptors.¹ This function appears to be restricted to certain species including rodents,^{1,2} but may be analogous to other hemostatic mechanisms involving platelet adhesion. For example, platelets bind to exposed collagen fibers following damage to blood vessel walls resulting in morphologic changes, release of α -granules and dense bodies, and platelet aggregation.³ More recently, platelets have been demonstrated to be directly involved in antibody-dependent cell-mediated cytotoxicity involving complement.⁴ Thus, mouse platelets are cytotoxic toward antibody-coated sheep erythrocytes in the presence of C5-deficient mouse serum. Furthermore, platelets from parasite-infected rats and from human subjects were able to efficiently kill *Schistosoma mansoni* larvae,^{5,6} a process involving parasite-specific IgE. Together these findings suggest that platelets may be primary mediators of immunologic destruction of systematically infecting microorganisms in both rodents and man. Furthermore, an immunologic role for platelets in cell turnover and perhaps even in tumor cell metastasis is possible.

A search for platelet cell surface molecules which may be involved in cytotoxic mechanisms revealed the presence of the leukocyte functional molecule LFA-1 on mouse platelets.⁷ In addition, the presence of complement receptor type 3 (CR3) was also demonstrated by the binding of M1/70 monoclonal antibody (MoAb) and by inhibition of immune adherence. In this study we show that C71/26 MoAb against Pgp-1, a cell surface glycoprotein present on bone marrow cells and cells of the granulocyte-macrophage lineage,⁸ also binds to mouse platelets and present evidence that this molecule may play a role in antibody-dependent platelet cytotoxicity involving complement.

MATERIALS AND METHODS

Animals and cell preparation. DBA/2, CBA/T6T6, C57B1/6, and (CBAXC57B1/6)F₁ mice were bred at Wellington Clinical School of Medicine. Platelets were prepared from whole cardiac

blood of CBA or F₁ mice, using heparin (25 U/mL) or trisodium citrate as an anticoagulant. Blood was centrifuged at 200 × g for ten minutes and platelet-rich plasma (PRP) aspirated with gentle stirring to increase the yield of platelets. Platelets were recovered by centrifuging PRP at 1,000 × g for five minutes and were washed in citrate-phosphate buffer pH 6.0 prior to use in fluorescent and solid phase assays and in immunoprecipitation. Platelet-poor plasma (PPP or plasma) was obtained either from whole blood centrifuged at 1,000 × g for ten minutes or as the supernatant from the purification of platelets from PRP. For cytotoxicity experiments platelets were resuspended in plasma from DBA mice.

Spleen, thymus, testis, bone marrow, and peripheral blood mononuclear cells isolated from the interface of Isopaque-Ficoll ($\rho = 1.09$) were prepared as washed single cell suspensions in phosphate buffered saline (PBS). Macrophages were prepared from peritoneal cells of CBA or F₁ mice chronically infected with 20 *Mesocostoides corti* larvae by repeated 1 × g sedimentation until the cell preparation contained greater than 90% macrophages. Liver, kidney, brain, and skeletal muscle were prepared by expressing tissue through a sieve and washing extensively with PBS.

MoAbs and antisera. C71/26.3.6 against Pgp-1, 121/7.7 against LFA-1 α , C71/16.2 against LFA-1 β , and 141/14.1 against T200 were obtained from Dr I. Trowbridge (Salk Institute, La Jolla, Calif); M17/5.1.9 and M17/4.4 against LFA-1 α and M1/70 against Mac-1 (CR3) were obtained from Dr T. Springer (Dana-Farber Cancer Institute, Boston); Dako-C3bR against human CR1 was obtained from Dakopatts, Copenhagen, Denmark; 2.4G2 against FcR2 from Dr J. Unkeless (Rockefeller University, New

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York); EDU-3 against platelet IIb/IIIa was from Dr R. Vilella (Hospital Clinico y Provincial, Barcelona, Spain); and 1G3 was derived in this laboratory by fusion of Sp2/0 plasmacytoma cells with spleen cells from a rat inoculated with mouse platelets. All MoAbs were rat IgG except C71/26.3.6, which was rat IgM, and Dako C3bR and EDU-3, which were mouse IgG. The MoAbs used, the proteins recognized, and source references are listed in Table 1.⁸⁻¹⁴

Antithymocyte serum (ATS) and antimacrophage serum (AMS) were prepared in New Zealand white rabbits as described previously⁷ and absorbed with an equal volume of erythrocytes.

Solid phase ELISA assay. Platelet suspensions (0.1 mL, 10^7 per mL) were distributed in flat-bottomed microtiter wells (Nunc, Roskilde, Denmark) and centrifuged at $1,000 \times g$ for five minutes. Glutaraldehyde (50 μ L, 0.25% in PBS) was added to each well without having removed the supernatant, and the plates centrifuged briefly at $150 \times g$ for five minutes. Fixation was continued at 20 °C for 10 to 15 minutes. The fixative was then removed, and the cells were washed with PBS, then with PBS containing 0.05% Tween-20 (Sigma Chemical Co, St Louis) and stored frozen in this buffer. Duplicate wells were treated with the antibody to be tested (25 μ L) followed by peroxidase-conjugated IgG sheep anti-rat IgG (Amersham, Sydney) diluted to 1:800. Peroxidase substrate (0.1% o-phenylenediamine, 0.012% H_2O_2 in 0.025 mol/L citrate-phosphate buffer, pH 4.7) was added, and the reaction terminated after ten minutes by adding an equal volume of 4N H_2SO_4 . Microtiter plates were read in an automated microplate reader (Dynatech, Singapore) at 490 nm using a reference wavelength of 410 nm.

Cell surface labeling and immunoprecipitation. Purified platelets (2 to 3×10^9) in PBS were labeled either with ^{125}I -sodium iodide (Amersham, Sydney) by the glucose oxidase modification¹⁵ of the lactoperoxidase technique¹⁶ or with ^3H by mild oxidation of sialic acid residues followed by reduction with ^3H -sodium borohydride.¹⁷ The radiolabeled cells were solubilized with 1% NP-40 in 50 mmol/L Tris-HCl buffer pH 7.2 containing 10 mmol/L phenylmethylsulfonyl fluoride and 0.3 U/mL aprotinin (Sigma). Following two preabsorptions with 0.2 vol of 10% v/v *Staphylococcus aureus* Cowan strain 1 (SaCl), the cell lysates were treated with either antiserum or MoAb followed by rabbit anti-rat IgG (Miles-Yeda, Rehovot, Israel) second antibody. The resulting immune complexes were immunoprecipitated with SaCl, washed, and boiled for six minutes in SDS sample buffer (0.1 mol/L Tris-HCl, 2% SDS)

Table 1. Antibodies Used in This Study

Antibody	Specificity*	Mol Wt of Reduced Protein	Source Reference
C71/26	Pgp-1	87,000–100,000	8
I42/5	Pgp-1	87,000–100,000	8
IM7	Pgp-1	87,000–100,000	8
I21/7	LFA-1 α	170,000	9
M17/5	LFA-1 α	170,000	10
M17/4	LFA-1 α	170,000	10
C71/16	LFA-1 β	95,000	9
M1/70	CR3(Mac-1)	160,000	11
Dako-C3bR	CR1	210,000	12
I41/14	T200	200,000	Personal Communication
EDU-3	IIb/IIIa	100,000–112,000	13
2.4G2	FcRII	47,000–60,000	14

* 1 Pgp-1, polymorphic glycoprotein-1; LFA-1, leukocyte function antigen-1; α , alpha subunit; β , beta subunit; CR3, complement receptor type 3; CR1, complement receptor type 1; T200, leukocyte common molecule; IIb/IIIa, platelet glycoprotein expressing fibrinogen receptor; FcRII, Fc receptor for IgG type II.

containing 5% mercaptoethanol. SaCl was removed by centrifugation and the supernatants electrophoresed on 6% to 12.5% gradient SDS-polyacrylamide gels after the method of Laemmli.¹⁸ Autoradiography was performed using Kodak XPR-1 X-ray film (Eastman Kodak, Rochester, NY) and calcium tungstate intensifying screens.¹⁹ For immunoprecipitation with ^3H -labeled material, the polyacrylamide gels were treated with 20% 2,5-diphenyloxazole in acetic acid²⁰ and were exposed against preflashed films.²¹

Immunoblotting. Cells or washed tissue (30 μ L packed volume) that had been expressed through a fine sieve were solubilized in 1 mL NP-40 solubilization buffer (see above) and 60 μ L samples electrophoresed on 5% to 15% gradient SDS-polyacrylamide gels as described above. The gels were transblotted onto nitrocellulose sheets (Schleicher and Schuell, Kean, NH) for 20 hours at 15 V (initial current of 700 mA) using the method of Towbin et al.²² Remaining protein binding sites on the membrane were blocked with 5% BSA at 20 °C for two hours prior to incubation with MoAb diluted 1:400 in Tris-buffered saline pH 7.5 containing 0.2% gelatin at 20 °C for 18 hours with shaking. The membrane was washed in buffer, then incubated with peroxidase-conjugated sheep anti-rat IgG (Amersham) for 4 hours at 20 °C. Color development was with 4-chloro-1-naphthol (60 mg/100 mL) in Tris-buffered saline.

Flow cytometry. Antibody binding to CBA mouse cells was determined using a fluorescence-activated cell sorter (FACS 420, Becton Dickinson, Sunnyvale, CA). Cell suspensions were treated with hybridoma supernatant, washed twice with PBS, then stained with affinity-purified IgG mouse anti-rat IgG coupled to fluorescein. Stained cells were analyzed directly or fixed with 10% paraformaldehyde in PBS and stored at 4 °C prior to analysis.²³ Cell populations such as lymphocytes and monocyte/macrophage cells were electronically "gated" following positive identification in low angle ν 90° scatter graphs and fluorescence intensity profiles measured on these cell populations using logarithmic amplification.

Immune adherence assay. Immune adherence was determined using platelet-dependent agglutination of antibody-coated sheep erythrocytes (EA) in the presence of mouse complement. This assay has been described previously⁷ and erythrocyte agglutination shown to be dependent on platelets, complement, and antibody-coated indicator cells and to be inhibited by EDTA and heparin. Platelet-rich plasma was prepared from cardiac blood of F_1 mice collected into siliconized tubes containing trisodium citrate as an anticoagulant. After centrifugation at $200 \times g$ for ten minutes, platelet-rich plasma was removed with gentle stirring to increase platelet yield, and the remaining suspension centrifuged at $1,200 \times g$ for five minutes to produce PPP. PRP was diluted 1:4 in PBS prior to use (platelet concentration 3×10^8 /mL). Platelets were mixed with PBS, or antibody (25 μ L final volume) for 30 minutes prior to addition of EA (25 μ L, 1% v/v). EA were prepared by coating sheep erythrocytes (2% v/v) with subagglutinating concentrations of rabbit hemolysin (Wellcome Reagents, Beckenham, England) (1:1,600 dilution in PBS). Microtiter plates were agitated gently at 20-minute intervals and agglutination assessed at 60 to 90 minutes. Under these conditions, and using diluted platelet-rich plasma (1:4), no hemolysis of EA was observed over the period of assay.

Platelet aggregation. Cardiac blood was used to prepare PRP and PPP as described above. Aggregation studies were performed at 37 °C under continuous stirring in a Corning (Corning, NY) EEL-169 platelet aggregometer. PRP (400 μ L) was mixed with antiserum or hybridoma supernatant (50 μ L) and aggregation initiated by addition of 50 μ L of 10 mmol/L ADP in saline. 100% transmission was determined with PPP.

Platelet cytotoxicity. Sheep erythrocytes from defibrinated blood were washed in Alsever's solution and stored at 4 °C. Erythrocytes were labeled with ^{51}Cr Chromium,⁴ washed three times with PBS, and coated with subagglutinating concentrations of rabbit hemolysin

(1/400 to 1/800) (Wellcome) prior to use. CBA or F_1 platelets were purified from PRP and suspended in a 1:4 dilution of plasma from DBA mice (2 to 6×10^9 platelets/mL). Aliquots of this platelet suspension were incubated with control NS1 supernatant or hybridoma supernatant ($25 \mu\text{L}$) at 20°C for 30 minutes prior to addition of ^{51}Cr -labeled antibody-coated sheep erythrocytes (0.25% v/v, $50 \mu\text{L}$). Incubation was continued at 37°C for three hours with gentle agitation at 15-minute intervals. Radioactivity released was determined in a γ -counter following centrifugation at $1,000 \times g$ for five minutes to remove unlysed cells. Specific lysis was determined relative to controls lacking platelets or using heat inactivated plasma from DBA mice and complete release of radioactivity with plasma from CBA or F_1 mice.

RESULTS

Identification and characterization of Pgp-1 on platelets. The presence of Pgp-1 on mouse platelets is shown in Fig 1 where the titration curve of C71/26 MoAb is compared with that of several other MoAbs against platelet-cell-surface glycoproteins, some of which are known to be associated with cell-mediated cytotoxic reactions (Table 1). Similar results have also been obtained by radioimmunoassay and by indirect immunofluorescence (results not shown). Immunoprecipitation of mouse platelets radiolabeled with ^{125}I -iodide (Fig 2) or ^3H -sodium borohydride (Fig 3) with C71/26 MoAb against Pgp-1 resulted in a broad band of radioactivity of mol wt 87,000 to 100,000. This band appeared to be different from the β -subunit of LFA-1 which immunoprecipitated a sharper band at mol wt 97,000. Immunoclearance experiments and peptide mapping showed these two glycoproteins to be quite distinct. Antiserum against mouse peritoneal macrophages immunoprecipitated LFA-1 from ^3H -labeled mouse platelets and a broad band of radioactivity which appeared to include Pgp-1. In contrast, antiserum against mouse thymocytes immunoprecipitated

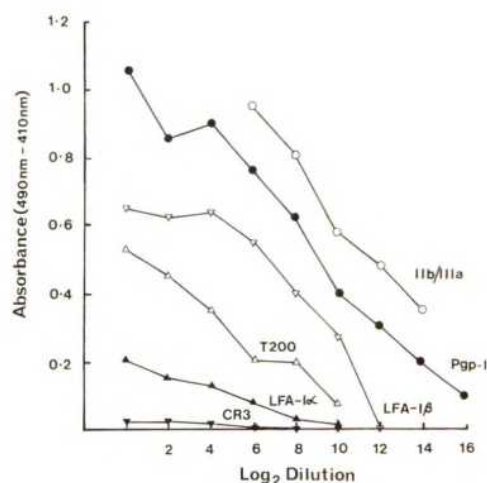


Fig 1. Binding of MoAbs against Pgp-1 and other functionally associated cell surface molecules to mouse platelets. Hybridoma supernatants C71/26 (Pgp-1), $\bullet$ — $\bullet$; C71/16 (LFA-1 β), ∇ — ∇ ; I41/14 (T200), Δ — Δ ; I21/7 (LFA-1 α), $\blacktriangle$ — $\blacktriangle$; and M1/70 (CR3), $\circ$ — $\circ$ were titrated against platelets using solid phase ELISA. Absorbance measurements (A490-410) were subtracted from controls using NS1 plasmacytoma culture supernatant.

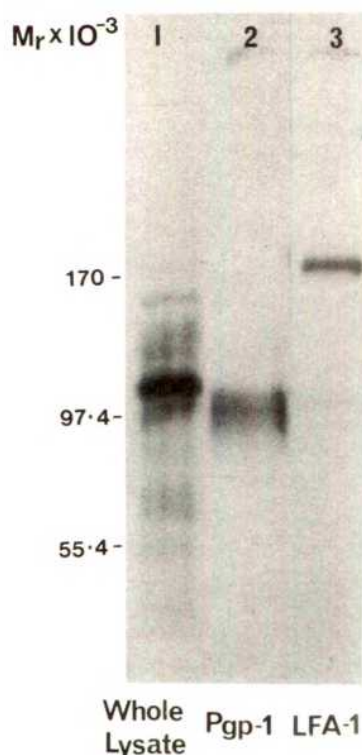
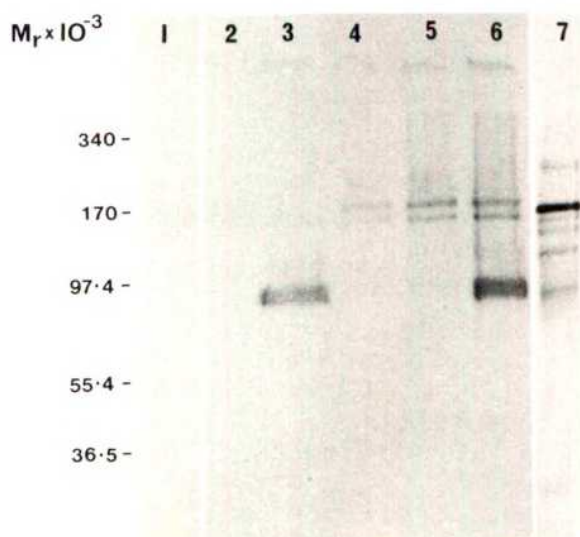


Fig 2. Immunoprecipitation of Pgp-1 from mouse platelets. Platelets surface-labeled with ^{125}I were lysed with detergent and immunoprecipitated with C71/26 (lane 2) or I21/7 (lane 3). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and the gels autoradiographed. For comparison, ^{125}I -labeled proteins from the whole platelet lysate are shown in lane 1. The two most prominent bands are glycoproteins IIb and IIIa.

LFA-1, but not Pgp-1 from ^3H -labeled platelets. A nonspecific band at mol wt 150,000 was also observed in immunoprecipitates from ^3H -labeled platelets. Immunoblotting allowed direct comparison of Pgp-1 molecules on platelets, macrophages, and spleen cells (Fig 4). Platelets showed two Pgp-1 bands at mol wt 87,000 and 97,000 whereas peritoneal macrophages showed the 87,000 mol wt band and a prominent additional band at mol wt 82,000. A faint Pgp-1 band was also observed on spleen cells at mol wt 89,000. Pgp-1 was not observed on thymocytes or on a mast cell precursor line (P-cell), or on the nonhematopoietic tissues tested, which included liver, kidney, brain, and testis. In other immunoblotting experiments, neutrophils and bone marrow cells showed a strong Pgp-1 band whereas erythrocytes, erythroid precursor cells from the spleen of phenylhydrazine-treated mice, eosinophils from *M. corti*-infected mice, and skeletal muscle tissue were negative.

The relative abundance of Pgp-1 on peripheral blood monocytes is demonstrated in Fig 5. More than 97% of peripheral blood monocytes and peritoneal macrophages expressed Pgp-1, the mean relative fluorescence (MRF) being 7.0 and 7.5, respectively. In contrast, lymphocytes from peripheral blood and from spleen showed heterogeneous fluorescence intensity profiles, with 20% to 30% of cells fluorescence negative and 5% to 10% strongly fluores-



Antibody: NRS Mac-1 Pgp-1 LFA-1 ATS AMS Whole Lysate

Fig 3. Glycosylation of platelet Pgp-1. Platelets surface-labeled with ^3H -sodium borohydride were lysed with detergent and immunoprecipitated with 1, control rabbit serum; 2, M1/70; 3, C71/26; 4, I21/7; 5, antithymocyte serum; and 6, antimacrophage serum. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and the gel autoradiographed. Lane 7 shows ^3H -labeled proteins in the whole platelet lysate.

cence positive (MRF = 6.5 to 7.0). The majority of lymphocytes were weakly positive (MRF = 2.5 to 5.0). Lymphocytes from the peritoneal cavity showed more homogeneous expression of Pgp-1, with 96% fluorescence positive with an intermediate MRF of 5.5. Platelets were also shown to be fluorescence positive for Pgp-1 by flow cytometry, the right shift of the fluorescent intensity profile with associated peak symmetry indicating that all platelets expressed Pgp-1.

Functional associations of Pgp-1 on mouse platelets. The presence of Pgp-1 on mouse platelets, monocytes, macrophages, and neutrophils and on subpopulations of

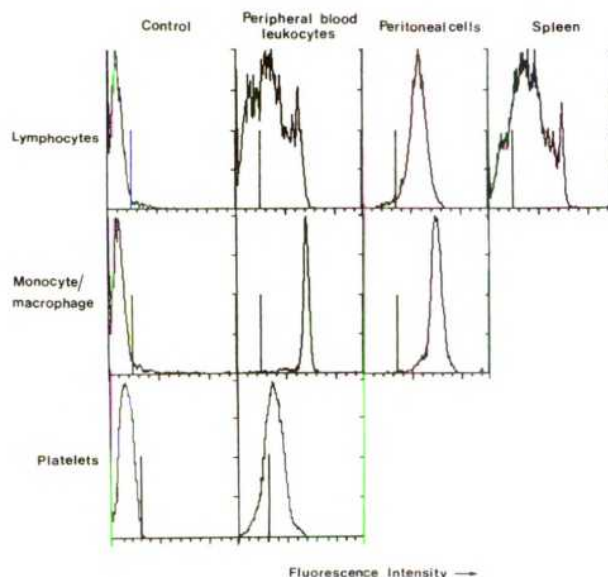


Fig 5. Comparison of binding of C71/26 MoAb against Pgp-1 to lymphocytes, monocytes, and platelets. Pgp-1 on gated cell populations in peripheral blood leukocytes and peritoneal cells and on spleen cells and platelets was determined by flow cytometry. Control cells were treated with NS1 plasmacytoma supernatant. A reference bar is included in each fluorescence intensity profile for comparative purposes.

lymphocytes suggests a role in some common function. Initial investigations of the effect of the MoAb C71/26 against Pgp-1 on platelet aggregation induced by adenosine diphosphate (ADP) showed no significant effect. Rodent platelets however are known to be involved in immune adherence,² a function which is also shared with phagocytic cells and lymphocytes. Thus, MoAbs against Pgp-1, CR3, CR1, FcR2, T200, and LFA-1 were tested for their ability to inhibit platelet adherence to antibody-coated sheep erythrocytes in the presence of complement. Figure 6 shows that M1/70 against CR3 extensively inhibited platelet immune adherence whereas Dako-C3bR against human CR1 inhibited to a titer of 1:8. At the highest concentration of antibody

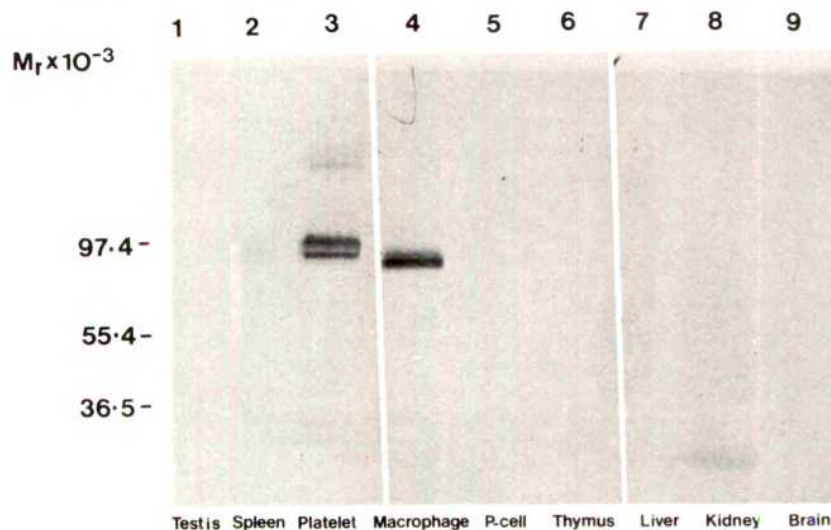


Fig 4. Immunoblotting of cell proteins with C71/26 MoAb against Pgp-1. Cells or washed tissue (0.5 μL packed volume equivalent) were solubilized in NP-40 buffer, electrophoresed on SDS-polyacrylamide gels, and transblotted onto nitrocellulose. Pgp-1 was detected with C71/26 MoAb, peroxidase-conjugated second antibody, and 4-chloro-1-naphthol.

Fig 6. Effect of MoAbs against Pgp-1 and other functionally-associated cell-surface molecules on platelet immune adherence. Platelet-rich plasma was diluted and incubated with antibody prior to the addition of opsonized erythrocytes. Inhibition of red cell agglutination is seen as a greatly reduced ability to form red cell buttons.

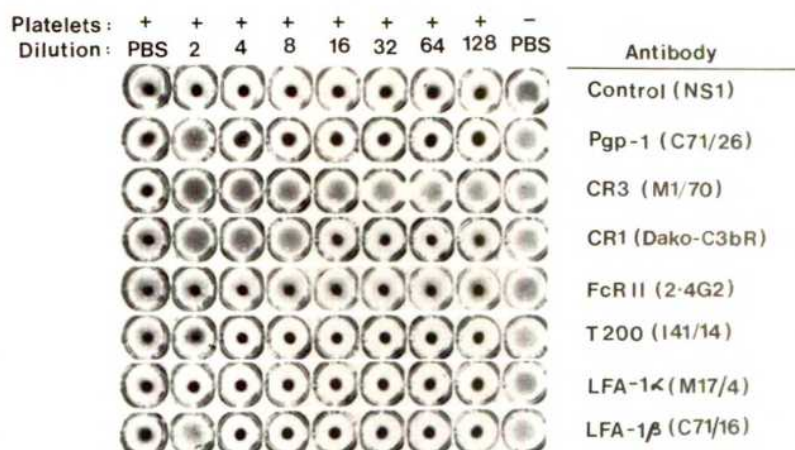


Table 2. Effects of MoAbs Against Pgp-1 and LFA-1 on Platelet Cytotoxicity

Plasma	Platelets	Antibody Specificity	^{51}Cr Release (cpm $\pm$ SE)	% Specific Lysis	% Inhibition Specific Lysis
<i>Experiment 1</i>					
—	—	—	102 $\pm$ 2	—	—
F ₁	—	—	760 $\pm$ 10	100	—
DBA	—	—	124 $\pm$ 4	—	—
DBA	F ₁	NS1	214 $\pm$ 14	14	—
DBA	F ₁	C71/26 Pgp-1	130 $\pm$ 20	1	93
DBA	F ₁	I21/7 LFA-1 α	157 $\pm$ 12	5	63
DBA	F ₁	C71/16 LFA-1 β	135 $\pm$ 5	1	90
<i>Experiment 2</i>					
—	—	—	262 $\pm$ 39	—	—
F ₁	—	—	1,996 $\pm$ 74	100	—
DBA	—	—	300 $\pm$ 2	—	—
DBA	F ₁	NS1	732 $\pm$ 26	25	—
DBA	F ₁	C71/26 Pgp-1	538 $\pm$ 13	14	44
DBA	F ₁	I42/5 Pgp-1	407 $\pm$ 48	6	75
DBA	F ₁	IM7 Pgp-1	558 $\pm$ 69	15	40
DBA	F ₁	I21/7 LFA-1 α	626 $\pm$ 38	19	25
DBA	F ₁	C71/16 LFA-1 β	373 $\pm$ 14	4	83
<i>Experiment 3</i>					
CBA	—	—	1,444 $\pm$ 41	100	—
DBA	—	—	316 $\pm$ 26	—	—
DBA	CBA	NS1	664 $\pm$ 8	32	—
DBA	CBA	C71/26 Pgp-1	312 $\pm$ 10	0	101
DBA	CBA	M1/70 CR3	686 $\pm$ 46	34	—6
DBA	CBA	I21/7 LFA-1 α	306 $\pm$ 10	—1	103
DBA	CBA	M17/5 LFA-1 α	640 $\pm$ 40	30	7
DBA	CBA	M17/4 LFA-1 α	592 $\pm$ 8	25	21
DBA	CBA	C71/16 LFA-1 β	432 $\pm$ 16	11	67
<i>Experiment 4</i>					
CBA	—	—	1,131 $\pm$ 68	100	—
DBA	—	—	268 $\pm$ 6	—	—
DBA	CBA	NS1	1,080 $\pm$ 22	94	—
DBA	CBA	C71/26 1:4 Pgp-1	638 $\pm$ 52	43	61
DBA	CBA	1:8 Pgp-1	804 $\pm$ 74	62	40
DBA	CBA	1:16 Pgp-1	996 $\pm$ 22	84	17
<i>Experiment 5</i>					
CBA	—	—	1,764 $\pm$ 8	100	—
DBA(56 °C, 30 min)	CBA	—	354 $\pm$ 40	—	—
DBA	CBA	NS1	728 $\pm$ 22	26	—
DBA	CBA	I42/5 Pgp-1	377 $\pm$ 15	2	92
DBA	CBA	9F6 H2 Class 1	785 $\pm$ 18	31	—19

used, C71/26 against Pgp-1 inhibited aggregation. The possibility that this inhibition of immune adherence may have been caused by antibody-dependent agglutination of platelets was examined by phase contrast microscopy. The only antibody which caused significant platelet agglutination was C71/26, and this effect was only evident with undiluted hybridoma supernatant.

Another function held in common between most cells expressing Pgp-1 is antibody-dependent cell-mediated cytotoxicity involving complement. To test the involvement of Pgp-1, LFA-1, and CR3 in platelet cytotoxicity, a ^{51}Cr -release assay similar to that described by Soper and Winn⁴ was used. The characteristics of this assay and the effect of MoAbs against Pgp-1, LFA-1, and CR3 on complement-dependent platelet cytotoxicity are shown in Table 2. In five experiments platelet-dependent lysis of ^{51}Cr -labeled sheep erythrocytes varied between 14% and 94%. Specific lysis was determined relative to background release in the absence of platelets using C5-deficient plasma from DBA mice (Experiments 1 through 4) or in the presence of platelets using heat inactivated plasma from DBA mice (Experiment 5) and complete ^{51}Cr -release with plasma from CBA or F_1 mice. Under conditions where platelet agglutination was not observed, C71/26 against Pgp-1 inhibited lysis of antibody-coated erythrocytes by 44% to 100%. With either F_1 or CBA platelets, inhibition was greater under conditions which favored less complete target cell lysis. Other MoAbs against Pgp-1, 142/5, and IM7 also inhibited platelet cytotoxicity (Table 2, Experiments 2 and 5). In addition, 121/7 against LFA-1 α and C71/16 against LFA-1 β reproducibly inhibited platelet cytotoxicity, although two other MoAbs against different epitopes of LFA-1 α , that is, M17/5 and M17/4, showed little or no effect. Similarly, M1/70 against CR3 did not significantly affect platelet cytotoxicity. The possibility that platelet-bound antibodies activated and depleted complement components indirectly causing inhibition of platelet cytotoxicity is ruled out by the observation that complement-fixing MoAbs such as M17/5 (Experiment 3) and 9F6 (Experiment 5), which bound strongly to platelets,^{7,24} did not inhibit platelet cytotoxicity.

DISCUSSION

The polymorphic cell surface glycoprotein Pgp-1 was first described by Hughes and August²⁵ as a major antigenic component of mouse embryo 3T3 fibroblasts, but later was shown to be a constituent of myeloid and lymphoid cell membranes.^{8,26-28} We show here that Pgp-1 is also represented on purified mouse platelets and present evidence of a role for Pgp-1 in antibody-dependent cellular cytotoxicity mediated by platelets. In an enzyme-linked immunosorbent assay (ELISA), MoAbs against Pgp-1 and human glycoprotein IIb/IIIa showed high binding to mouse platelets relative to other MoAbs tested. Both the α - and β -subunits of LFA-1 and T200 were also present. These results confirm previous results which showed that EDU-3 cross-reacted with a small fraction of mouse platelets,²⁹ and that LFA-1⁷ and certain epitopes of the T200 family of cell surface glycoproteins³⁰ were also expressed on mouse platelets. Both immunoprecipitation and immunoblotting procedures indicated heterogeneity of the Pgp-1 molecule on platelets, immunoblotting resolving discrete molecular forms on both platelets and macrophages. A minor platelet protein of mol wt 180,000 detected by C71/26 MoAb by immunoblotting has not been identified, but may represent a precursor form of Pgp-1, possibly of cytoplasmic origin as it was not detected by surface-labeling procedures. The Pgp-1 glycoprotein on platelets did not correspond with any of the major surface-labeled glycoproteins (Figs 2 and 3) although possible identity with platelet glycoprotein IV³¹ is suggested by similar mol wts and by lack of mol wt shift following complete reduction (results not shown).

The distribution of Pgp-1 on mouse splenic lymphocytes (Fig 5) shows close similarity to previously published results²⁸ in which the majority of cells exhibited low relative fluorescence. However, we have detected a minor population (5% to 10%) of more brightly fluorescent lymphocytes in both splenic and peripheral blood. These results were supported by the immunoblotting studies which showed low levels of Pgp-1 on spleen cells relative to macrophages and platelets (Fig 4). Flow cytometry also indicated low relative fluorescence per platelet, a result which reflects the small surface area of the platelet compared to lymphocytes and macrophages.

Despite the isolation and biochemical and genetic characterization of Pgp-1,^{8,25-28,32,33} its function has remained elusive. The description of Pgp-1 on platelets provided a system in which to test several functional associations. Initial experiments indicated no effect of antibodies against Pgp-1 on platelet aggregation and a marginally significant inhibition of immune adherence of platelets to antibody-coated sheep erythrocytes in the presence of complement (Fig 6), an effect that correlated with antibody-induced platelet agglutination. That immune adherence is mediated through complement receptors is suggested by the fact that MoAbs against both CR3 (Mac-1) and human CR1 inhibited platelet-erythrocyte agglutination under conditions where no platelet agglutination was observed. Using C5-deficient plasma from DBA mice, platelets were shown to lyse antibody-coated sheep erythrocytes. Specific lysis varied between 14% to 94% and was determined relative to controls lacking platelets and to complete release via the complement cascade using plasma from F_1 or CBA mice. The reason specific lysis varied in different experiments is not fully understood, but possibly relates to the state of platelet activation in individual experiments, to the quality of the target erythrocytes, to the mouse strain used to prepare platelets, and to the nature and frequency of shaking which has been shown to influence platelet cytotoxicity.⁴ The experiments described in Table 2 have used purified CBA or (CBAXC57B1/6) F_1 platelets in the presence of C5-deficient plasma from DBA mice to effect cytotoxicity of antibody-coated erythrocytes. Platelets were washed prior to use so that plasma carryover was at least two orders of magnitude lower than that required to effect cytotoxicity by the complement cascade. Furthermore the presence of plasma from DBA mice is necessary to cause platelet-dependent cytotoxicity; omission of plasma or heat-

treatment of the plasma negates the killing effect. In some experiments we have observed cytotoxicity with PRP from DBA mice, but in general cytotoxicity is lower and the effect more variable than with CBA or F₁ platelets. Inhibition of platelet cytotoxicity with C71/26 MoAb against Pgp-1 was highly significant within each experiment, more extensive at low specific lysis with a particular mouse strain, and titratable (Table 2). Furthermore, the possibility that the inhibition of platelet cytotoxicity seen with C71/26 MoAb was caused by platelet agglutination by virtue of its IgM isotype was ruled out both visually and by using other Pgp-1 MoAbs of IgG2a and IgG2b isotypes (Table 2).²⁸ No significant effect of M1/70 MoAb against CR3 was observed. In contrast, MoAbs against LFA-1 produced variable inhibition of platelet cytotoxicity, I21/7 against the α -subunit and C71/16 against the β -subunit inhibiting specific lysis by 25% to 103%, M17/4 inhibiting by 21%, and M17/5 showing no inhibition. Thus, certain epitopes on both Pgp-1 and LFA-1 on platelets appear to be involved in or closely associated

with the cytolytic process. The variability of inhibition of cytotoxicity observed with MoAbs against LFA-1 has been observed previously with CTL-mediated killing in both human³⁴ and mouse^{10,35} studies. Inhibition of cytolysis appeared to be dependent on the target cell used³⁵ and no correlation of the effect of LFA-1 monoclonal antibodies on platelet cytotoxicity and CTL-mediated killing were evident. It will be of interest to determine the effect of MoAbs against Pgp-1 on other antibody-dependent cytotoxic events—for example, those involving macrophages and neutrophils, which have both been shown to express Pgp-1. Furthermore, it would also be of interest to investigate functionally human cells, which may also express Pgp-1.²⁸ In this context, human platelets have recently been shown to exert cytotoxic effects on tumor cells.³⁶

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Cloning and Characterization of Platelet Factor 4 cDNA Derived From a Human Erythroleukemic Cell Line

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We report the isolation of a platelet factor 4 (PF4) cDNA clone from a λ gt11 expression cDNA library which was derived from a human erythroleukemic (HEL) cell line. The sequence of the DNA insert includes the 3'-untranslated region, the entire amino acid coding region for the mature PF4 protein, and a 5' region containing coding information for an additional 18 amino acids. In addition, supplemental genomic DNA sequencing shows that the full-length leader sequence is 30 amino acids long plus an initial methionine and codes for a hydrophobic signal-like sequence which is probably involved in transmembrane transport. A single species mRNA of approximately 800 nucleotides was detected on blots of HEL cell poly(A) + RNA using a labeled PF4 cDNA probe. The human PF4 leader sequence shares

some DNA, but no amino acid, homology with the 15 amino acids at the N-terminus of mature bovine PF4, suggesting rapid divergence in this region of PF4 between these two species. Sequence comparison of the coding regions of mature PF4 and γ IP-10, a protein induced in a variety of cells following treatment with γ -interferon, shows a corrected divergence of 76%. The divergence of a common ancestor protein into PF4 and γ IP-10 may have accompanied the development of sophisticated immune and coagulation systems in vertebrates. The availability of cDNA and genomic DNA information for these genes in other species will be useful in studying the evolution of the coagulation and immune systems.

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Platelet factor 4 (PF4) is made in megakaryocytes and packaged into α granules for release during platelet activation.¹⁻⁴ PF4 strongly binds to heparin and related glycosaminoglycans.^{5,6} A major physiologic role of PF4 may be the neutralization of heparin-like molecules in the serum and on endothelial cell surfaces, thereby decreasing antithrombin III activity and allowing coagulation to proceed.⁷ PF4 also binds to platelet membranes, activating coagulation at low agonist concentrations⁸; acts as a chemotactic factor for neutrophils, monocytes, and fibroblasts^{9,10}; stimulates the release of histamines by basophils¹¹; and substitutes for fibronectin in the adherence and spreading of cells on surfaces in vitro.¹²

Human PF4 contains 70 amino acids and has four cysteine residues; these participate in the formation of intramolecular disulfide bonds.¹³⁻¹⁶ A larger precursor protein for PF4 in rabbits was suggested from analysis of pulse-labeled proteins from rabbit megakaryocytes; however, pulse-chase experiments were not consistent with a precursor-product relationship between the larger protein and PF4.⁴

Bovine PF4 is the only PF4 other than human for which an amino acid sequence has been determined.¹⁷ The bovine protein is 88 amino acids long, with 70% homology to human PF4 in the region of overlap. The most striking difference between the two is the presence of an additional 15 amino acids at the N-terminus of bovine PF4.

PF4 is a member of a family of small proteins that are released from cells after stimulation and act as secondary signals in the related processes of coagulation, inflammation, and tissue repair. PF4 shares amino acid sequence homology with β -thromboglobulin (β TG)¹⁸ and γ IP-10.¹⁹ β TG is also a megakaryocyte/platelet-specific protein which is stored in α granules. A comparison of human β TG and PF4¹⁸ shows correspondence at 42 of the 81 amino acid residues, with amino acid differences at another 11 residues that can be attributed to single-base replacements at the DNA level. The four cysteine residues occupy the same relative position in the two molecules. γ IP-10 is synthesized and released from several cell lines following γ -interferon stimulation. Over a 62 amino acid region, γ IP-10 and PF4 have 22 identical amino acids and 26 amino acids that can be accounted for by

single-base replacements at the DNA level. In addition, hydrophobic profiles and placement of cysteine residues are similar, suggesting evolution from a common precursor.

The study of the evolutionary relationships and functional roles of these small proteins would be enhanced by isolation of the genes for PF4 and β TG. The ability to isolate and study genes which are expressed only in megakaryocytes and platelets was hampered until recently by the difficulty of isolating sufficient mRNA to establish a cDNA library. The discovery that a human erythroleukemic (HEL) cell line expresses a number of megakaryocyte-like proteins as determined by electron microscopy and immunologic detection²⁰ prompted us to establish a cDNA expression library using mRNA from these cells. We now describe the isolation and characterization of a human PF4 cDNA clone from this library and present evidence for the existence of a PF4 precursor protein in humans. We also present new observations on sequence homology at the DNA and amino acid levels between human PF4, bovine PF4, and γ IP-10.

MATERIALS AND METHODS

Cell line and preparation of a cDNA expression library. The HEL cell line was kindly provided by P. Martin, Seattle.²¹ It was grown and induced with 1.25% (v/v) dimethylsulfoxide, and total

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RNA was prepared using guanidinium thiocyanate (Fluka, Ronkonkoma, NY) and banding onto a cushion of cesium chloride.²² Total RNA was precipitated with ethanol at -70°C , and poly(A)⁺ RNA isolated by passage through an oligo-dT cellulose column (Collaborative Research, Inc, Bedford, MA). A cDNA library was constructed in λgt_{11} , as described,²³ except that double-stranded cDNAs were fractionated by size twice on Sepharose CL4B prior to cloning to enrich for molecules greater than 300 bp in length. The HEL cell library contained 1.5×10^6 recombinants, with an average insert size of 1.7 ± 0.8 kb.

Library screening. The library was screened with a commercial human PF4 antibody prepared in goats (Atlantic Antibodies, Scarborough, ME).²⁴ Positive plaques were detected, isolated, and purified using a biotinylated rabbit anti-goat antibody, which was then coupled to an avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Inc, Burlingame, CA), with detection of complexes facilitated by localization of bound peroxidase using the chromogen 4-chloro-1-naphthol.

Characterization of positive plaques. Phage DNA was purified from positive plaques, digested with *EcoRI* (Bethesda Research Laboratories, Gaithersburg, MD, (BRL)), and the insert isolated by electroelution (International Biotechnologies, Inc, New Haven, CT) following size fractionation on agarose gels. The insert was mixed with 200 ng of *EcoRI*-digested RF M13mp19 phage, precipitated in ethanol, pelleted, ligated, and transfected into *E. coli* JM107.²⁵ Double-stranded DNA was purified from clear plaques and characterized on gels following *EcoRI* digestion. Single-stranded DNA was also prepared, and phage with inserts in opposite orientation was identified following gel analysis of S1-nuclease-resistant heteroduplexes. We determined the DNA sequence of single-stranded DNA from phage harboring the insert in the two different orientations by the dideoxy sequencing technique using the universal M13 primer (BRL).^{26,27}

Isolation of additional human cDNA and genomic PF4 clones. Double-stranded PF4 insert was labeled with $\alpha\text{-}^{32}\text{P}$ -dCTP (Amersham, Arlington Heights, IL) using *E. coli* DNA polymerase (BRL) and calf thymus DNA primers.²⁸ This probe was used to rescreen the HEL cell cDNA library as well as a human genomic DNA library which was constructed by partial *Sau3A* digestion of genomic DNA followed by cloning into EMBL 3A as previously described.^{29,30} Positive clones were plaque purified, and appropriate inserts subcloned into M13 bacteriophage as described above.

Characterization of PF4 mRNA from HEL cells. ^{32}P -labeled PF4 cDNA probe was also hybridized to Nytran (Schleicher and Schuell, Keene, NH) blots of agarose gel-fractionated, formaldehyde-denatured HEL cell poly(A)⁺ RNA.²⁸ RNA size was determined relative to ^{32}P -labeled DNA markers made by end-labeling Hae III digests of ϕX174 phage (BRL).

Computer analysis. PF4 and $\gamma\text{IP-10}$ cDNAs were analyzed for DNA homology with the ALIGN program on BIONET.³¹ In addition, the two proteins were compared by the FASTP program³² which aligns sequences on the basis of an amino acid replaceability matrix, giving higher scores to those replacements which occur frequently in evolution.

RESULTS

Isolation and characterization of a PF4 cDNA clone. Two positive clones, each containing approximately 400-bp inserts, were found while screening 1.2×10^5 recombinants. One insert was isolated, subcloned into M13, and the DNA sequence determined (Fig 1). This 409-bp insert contains a 54-bp 5'-leader sequence followed by the entire coding sequence of PF4, an in-frame termination codon (TAG), a

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-7      1      48
ccgcagc  ATG AGC TCC GCA GCC GGG TTC TGC GGC TCA GGC CCC GGG CTG CTG TTC
          MET ser ser ala ala gly phe cys ala ser arg pro gly leu leu phe

          102
CTG GGG TTG CTG CTC CTG CCA CTT GTG GTC GGC TTC GGC AGC GCT GAA GCT GAA
leu gly leu leu leu leu pro leu val val ala phe ala ser ala glu ala glu

          156
GAA GAT GGG CAC CTG CAG TGC CTG TGT GTG AAG ACC ACC TCC CAG GTC CGT GGC
glu asp gly asp leu gln cys leu cys val lys thr thr ser gln val arg pro

          210
AGG CAC ATC ACC AGC CTG GAG GTG ATC AAG GCC GGA CCC CAC TGC CCC ACT GCC
arg his ile thr ser leu glu val ile lys ala gly pro his cys pro thr ala

          264
CAA CTG ATA GCC ACG CTG AAG AAT GGA AGG AAA ATT TGC TTG GAC CTG CAA GCC
gln leu ile ala thr leu lys asp gly arg lys ile cys leu asp leu gln ala

          321
CCG CTG TAC AAG AAA ATA ATT AAG AAA CTT TTG GAG AGT TAG ctactagctgacctac
pro leu tyr lys lys ile ile lys lys leu leu glu ser TER

          392
gtgtgtgcatttgcctatagcactacttctttttccagtttcaactctgaagaaacttctgata

          448
tttgtgttatccctatgatttttaataacaaataaatcaaaaaaataaaaaa

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Fig 1. DNA sequence of human PF4 cDNA. The sequence shown is derived mainly from the 409 bp PF4 cDNA clone. The 5'-untranslated region sequence and the first 35 nucleotides of the coding region were obtained from a PF4 genomic DNA clone. The amino acid sequence for the leader sequence and mature PF4 are included. The arrowhead indicates the N-terminus of mature PF4. The DNA sequence for the coding region is in capital letters, and the partial 5'- and complete 3'-untranslated regions are shown in lower-case letters. Two potential polyadenylation signal sequences are underlined.

126-bp 3'-untranslated region containing two potential polyadenylation signals, and an oligo-dA tail of 16 adenosine residues located 11 bp 3' to the first polyadenylation sequence. The 54-bp 5' leader contains an open reading frame for 18 amino acids and no in-frame chain-initiating sequence for methionine (ATG), indicating that human PF4 is derived by proteolytic processing from a precursor with at least 18 additional amino acids at the N-terminus.

Other PF4 cDNA clones isolated from the expression library provided no additional 5' DNA sequence information. We therefore determined the remaining leader sequence from partial sequencing of a human genomic PF4 clone. The full-length leader is 30 amino acids long, preceded by a methionine residue and a 5'-noncoding sequence which contains a consensus sequence for a translational initiation site.³³ The 31-amino-acid leader sequence contains a 17-amino-acid hydrophobic core.

The size of PF4 mRNA was estimated to be approximately 800 nucleotides (n) (Fig 2), which is 350 n larger than the sequence shown in Fig 1, suggesting (assuming a poly A tail of 100 to 200 n) a 5'-untranslated region of approximately 200 n.

Abundance of PF4 mRNA in HEL cells. A variety of methods was used to estimate the abundance of PF4 mRNA in HEL cells. Two PF4 cDNA clones were detected from 1.2×10^5 recombinants using the antibody screen. Since only one of six possible reading frames leads to proper protein expression in this system, the abundance of PF4 mRNA is estimated at 0.01% ($12/[1.2 \times 10^5]$). Visual comparisons of lighter exposures of the autoradiograph in Fig 2 and other autoradiographs of known amounts of PF4 cDNA insert and mRNA blotted to Nytran (data not shown) suggest again

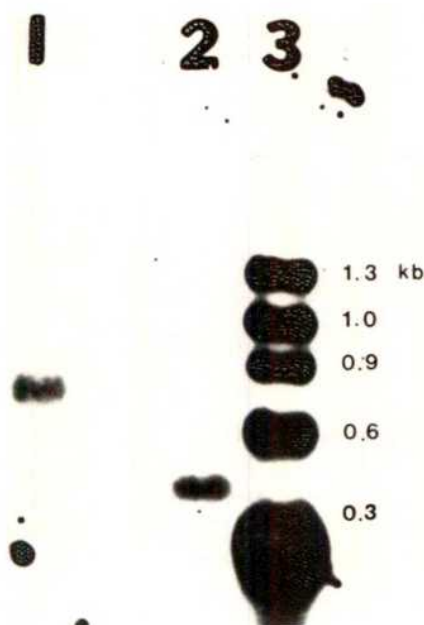


Fig 2. Size of PF4 mRNA in HEL cells. Autoradiograph of a nitrocellulose blot hybridized to labeled PF4 insert. Lane 1 = 0.8 μ g HEL cell poly A (+) RNA. Lane 2 = 10 pg of the 408-bp PF4 insert. Lane 3 = ^{32} P end-labeled ϕ X Hae III DNA markers.

that the frequency for PF4 mRNA in HEL cell mRNA is $\approx 0.01\%$.

Comparison between human PF4, bovine PF4, and human γ IP-10. The amino acid sequence of bovine PF4 is homologous to human PF4 over much of the molecule; however, it differs by the addition of 15 amino acids at its N-terminus that are not present in human PF4.¹⁷ Comparing the human PF4 leader with the amino acid residues at the N-terminus and the deduced DNA coding sequence of bovine PF4 reveals sequence similarity at the DNA, but not the amino acid level (Fig 3). There is a 30-bp region of 63% DNA homology, underlined in Fig 3. While DNA homology suggests that the N-terminus of bovine PF4 and the leader of human PF4 may be related, no significant amino acid homology exists, apparently secondary to nucleotide deletions and insertions.

A previous study¹⁹ using the FASTP protein analysis program³² reported extensive homology between the mature human PF4 and γ IP-10. Using the same program, we compared the human PF4 precursor protein sequence determined from our cDNA clone with the protein γ IP-10, and

found the same match previously reported.¹⁹ No new N-termini amino acid homology was detected.

Human PF4 and γ IP-10 cDNA sequences were also compared at the DNA level using the ALIGN program.³¹ DNA sequence similarity was found at the 3' end of the coding regions. This similarity begins at the 19th amino acid (valine) of the mature PF4 and the 18th amino acid (valine) of the processed γ IP-10 and extends for approximately 100 bp downstream, depending on the parameter settings (data not shown). A comparison of the 3'-untranslated regions revealed no further DNA sequence similarity. A comparison of the leader regions of the two proteins reveals a 22-bp region (underlined in Fig 3) with 73% similarity, suggesting that this region of the two proteins may have evolved from a common sequence. Further studies of these genes in other species should be helpful in resolving the evolutionary relationship between the leader regions of these proteins.

Analysis of the DNA sequences coding for the mature PF4 and γ IP-10 reveals 43% sequence similarity, with 47% similarity for the first nucleotide of the coding triplets, 56% for the second, and 25% for the third. Divergence for nonsynonymous substitutions (those causing amino acid changes) between these genes was 76%, including a correction for multiple substitutions at the same nucleotide.³⁵

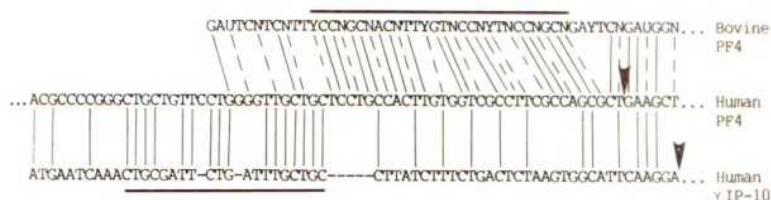
DISCUSSION

The PF4 cDNA is the first probe isolated for a protein whose expression is restricted to the megakaryocyte/platelet lineage. These studies confirm immunologic and morphologic data that HEL cells express megakaryocytic/platelet-like properties.²⁰ We are presently pursuing the isolation and characterization of additional cDNA and genomic clones for platelet-specific proteins in order to better understand their biological properties and coordinated expression during megakaryocyte development.

Attempts to understand megakaryocytic development from an undifferentiated stem cell have been limited to morphologic, cytochemical, and immunologic studies. The PF4 cDNA probe will now facilitate detection of changes at the DNA and transcriptional levels prior to accumulation of protein during normal megakaryocyte development, as well as in leukemias and myelofibrosis syndromes characterized by aberrant expression of megakaryocyte-like cells.

Our results demonstrate the presence of a human PF4 leader sequence of 30 amino acids whose structure resembles other known signal sequences involved in transmembrane transport.³⁴ These results suggest that the PF4 leader may be

Fig 3. Similarity between the leader sequence for human PF4, the N-terminus of bovine PF4, and the leader sequence of γ IP-10. The bovine PF4 DNA sequence was deduced from its amino acid sequence. U = purine, Y = pyrimidine, and N = any nucleotide. A line represents identity, a dashed line represents potential identity in cases where ambiguity exists in the triplet coding sequence. A 30-n region of similarity between human and bovine PF4 is overlined, and a 22-n region of similarity with γ IP-10 is underlined. Arrowhead indicates cleavage sites for the generation of the mature protein.



involved in packaging PF4 into α granules, followed by removal by proteolysis. Leader sequences of other proteins which are targeted to specific subcellular organelles have been shown to be critical for the proper localization of these proteins. The PF4 leader sequence differs from leader sequences of mitochondrial proteins³⁶ by lacking arginine or lysine residues thought to be critical for targeting to the mitochondrion. Comparison of human PF4 leader with the leader sequence of the γ chain of fibrinogen³⁷ also reveals neither amino acid nor hydrophobic regions of homology. The γ chain of fibrinogen is made in the liver, but is also thought to be synthesized in megakaryocytes and stored in α granules.² The leader sequence for this protein contains 26 amino acids, consisting of 12 hydrophilic residues and no long stretch of hydrophobic amino acids. Whether this leader sequence is also involved in targeting γ -fibrinogen to α granules is not known. Perhaps an alternative leader sequence is utilized for γ -fibrinogen synthesized in the megakaryocyte. Determination of leader sequences for other platelet proteins destined for localization in α granules should further our understanding of the relationship between leader sequence and cellular localization. The N-termini of human and bovine PF4 appear to have evolved from a common DNA sequence, yet code for divergent amino acid sequences, with the bovine N-terminus lacking the hydrophobic core that is characteristic of many signal sequences. Ultrastructural studies show that bovine platelets lack a cytoplasmic canalicular system,³⁸ and the sequence differences between PF4 in these two species may possibly be a reflection of basic differences in membrane organization requiring different modes of PF4 transport and release.

It appears that the three human proteins, PF4, β TG,³⁹ and γ IP-10, are generated by cleavage at comparable sites in

their precursors, suggesting divergence from a primordial gene. Since many genes appear to have functional subunits divided into separate exons,^{40,41} we suggest that the cleavage site also represents the boundary of two genomic exons, one coding for the leader and the other for the small signal protein. Preliminary sequence analysis of our genomic PF4 clone suggests that this is the case for human PF4.

Comparison of DNA sequences coding for mature PF4 and γ IP-10 reveals significant divergence. The rate of nonsynonymous divergence for mammalian genes varies considerably, ranging from 0.004×10^{-9} to 2.80×10^{-9} substitutions per site per year,⁴² and also varies among different species⁴³ and in different evolutionary epochs.⁴⁴ Therefore, the actual time of divergence can only be estimated for the PF4 family of genes. It is of interest that interferon-like substances have only been detected in vertebrates, whose divergence from invertebrates is estimated to have occurred approximately 500 million years ago,⁴⁵ equivalent to a nonsynonymous rate of divergence of 1.52×10^{-9} for PF4 and γ IP-10. The divergence of a primordial protein into PF4 and γ IP-10 may have accompanied the development of sophisticated immune and coagulation systems in vertebrates. The availability of cDNA and genomic probes for these genes in other species will be useful in studying the evolution of the coagulation and immune systems.

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Steady State Levels of Factor X mRNA in Liver and Hep G2 Cells

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In order to examine the control of human factor X biosynthesis we have molecularly cloned the cDNA and investigated the expression of the Factor X gene. A recombinant clone of approximately 1100 base pairs in length containing the sequence of factor X was identified in a λ gt11 human liver cDNA library by screening with polyclonal antibodies. One plaque was selected and confirmed for specificity with a mixture of five factor X specific monoclonal antibodies (MoAbs). A partial nucleic acid sequence of the 5' end of the cDNA corresponded to the described amino acid sequence between residues 41 and 56 of the light chain of factor X. Northern blot analysis of RNA from human liver and the hepatoma cell line, Hep G2, identified the factor X mRNA as a single molecular species of approximately 1700

bases. Cell lines which do not secrete factor X did not contain factor X mRNA indicating restriction of transcription to hepatocytes. Slot-blot hybridization analysis of factor X and actin mRNA demonstrated no change in the levels of total or specific factor X mRNA in Hep G2 cells following treatment with warfarin or vitamin K. We conclude that modulation of factor X production by these drugs, known to influence γ -carboxylation and total factor X secretion by these cells, is mediated by changes in posttranscriptional events rather than by effects on the steady state levels of factor X mRNA.

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THE VITAMIN K-DEPENDENT coagulation proteins (factors VII, IX, and X, prothrombin, and proteins C and S) in human plasma are a family of molecules that are characterized by amino acid sequence homology and by unique γ -carboxyglutamic acid (gla) residues located in the amino-terminal domain.^{1,2} These gla residues, which are necessary for function resulting from calcium and phospholipid binding and conformational ordering of the proteins, are formed by a posttranslational modification step in which CO₂ is added to selected glutamic acids. The process also requires O₂, vitamin K, and the carboxylating enzyme system (carboxylase/reductase).^{2,3} Warfarin inhibits the vitamin K epoxide reductase which catalyzes the conversion of vitamin K, 2,3-epoxide to vitamin K.^{2,3} Administration of this drug to individuals decreases the enzymatic activity and concentration of all known vitamin K-dependent proteins.⁴⁻⁹ Vitamin K and warfarin have also been observed to modulate the levels of γ -carboxylated coagulation proteins secreted into the media from cultured cells derived from human and

rat hepatomas.¹⁰⁻¹² Decreased secretion of these proteins in response to warfarin treatment may be attributed either to a decrease in the rate of synthesis or to an increase in catabolic rate of the resulting partially modified proteins. Previous results from our laboratory suggest that the effect of warfarin on prothrombin and factor X levels is probably on synthesis.¹² Vitamin K may reverse this by increasing the rate of secretion from intracellular pools, as seen with rat prothrombin.¹⁰ Alterations in the rate of synthesis (transcription or translation), secretion, and turnover have been suggested as potential mechanisms responsible for changes in the concentration of proteins of this family in response to warfarin or vitamin K.¹⁰⁻¹³

Factor X is a 59,000 mol wt two-chain glycoprotein with a heavy (42,000) and a disulfide-coupled light (17,000) chain.^{14,15} The light chain contains 12 gla residues, and the heavy chain contains the active site. Studies using rat hepatocytes and rat and human hepatoma cells have indicated that factor X is synthesized as a single chain molecule by the liver.^{12,13,16} Analyses of cDNA clones from bovine and human liver libraries indicate that the factor X gene is transcribed as a single mRNA species consistent with the protein synthesis studies.¹⁷⁻¹⁹

The human hepatoma cell line (Hep G2) produces a variety of plasma proteins²⁰ including most of the vitamin K-dependent protein molecules.^{12,21} These proteins are rapidly secreted by the cell and resemble their plasma protein counterparts structurally, immunologically, and functionally.^{11,12,21} Further this cell line responds to warfarin and vitamin K by modulating the activity and concentration of the gamma carboxylated proteins.^{12,21} Decreased levels of prothrombin were noted 24 hours after exposure to warfarin, which was attributed to a reduced synthetic rate.¹² Decreased levels of factor X and the other vitamin K-dependent factors can be shown after they accumulate during a more prolonged culture period.^{12,21} One possible explanation for these observations would be an alteration in the transcriptional rate of the genes encoding these proteins. We have identified a partial cDNA recombinant clone for factor X from a λ gt11 expression library and have used it as a probe to characterize factor X mRNA in the Hep G2 cell line and human liver. Slot-blot analysis was used to investigate

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the effects of vitamin K and warfarin on the expression of the factor X gene in Hep G2 cells by measuring the steady state levels of factor X mRNA.

MATERIALS AND METHODS

Materials. The human hepatoma cell line Hep G2 was provided and characterized by Drs Barbara B. Knowles and David P. Aden of the Wistar Institute, Philadelphia.²⁰ The cDNA probe for human cytoplasmic actin was kindly provided by Dr Elaine Fuchs of the University of Chicago.²²

Vitamin K (Aqua Mephyton) was obtained from Merck Sharp & Dohme (West Point, Pa). Restriction enzymes were from International Biotechnologies, Inc. (New Haven, Conn), and DNase I from Promega Biotec (Madison, Wis). Guanidinium isothiocyanate was purchased from Fluka Chemical Corp (Hauptpauge, NY), hydroxystilbamidine isothionate (HBI) was from Merril National Labs (Cincinnati, Ohio), and cesium trifluoroacetate (CsTFA) was from Pharmacia Fine Chemicals (Piscataway, NJ). Nucleic acid grade phenol, agarose, and formamide were obtained from Bethesda Research Laboratories (Bethesda, Md). Glyoxal was from Eastman Kodak Co. (Rochester, NY), and methyl mercury hydroxide from Alfa Products (Danvers, Mass). Polyadenylic acid and salmon sperm DNA were purchased from P.L. Biochemicals (Milwaukee). Quick-seal centrifuge tubes were purchased from Beckman Instruments, Inc (Palo Alto, Calif). Nitrocellulose and slot-blot apparatus (Minifold II) were purchased from Schleicher and Schuell, Inc (Keene, NH). Other enzymes and reagents were obtained from Sigma Chemical Co (St. Louis, Mo). Radioactive isotopes were from Amersham Co (Arlington Heights, Ill).

Cell culture. The effects of vitamin K and warfarin on the levels of factor X mRNA in the Hep G2 cell were investigated using 3×3 fixed factor analyses of variance without replication.²³ Conditions for growing and maintaining Hep G2 cells in culture were as described.¹² For experimental studies T-75 flasks were seeded with 1×10^6 cells and grown for seven days in Eagle's minimal essential media (MEM) and 10% heat-inactivated fetal calf serum (FCS). The cultures were washed and incubated approximately 30 minutes in serum-free MEM. The media was replaced with 12 mL of MEM (control) or in MEM supplemented with 10 μ g/mL vitamin K or 1 μ g/mL of the warfarin derivative, 3 (α -acetylbenzyl)-4-hydroxycoumarin. After the first 24-hour incubation, the cultures were washed and incubated with serum-free MEM as described above. One flask from each treatment group received media, media supplemented with vitamin K, or media with warfarin, after which the cells were incubated another 24 hours. The cells were then harvested, and total RNA isolated as described below.

Cloning of factor X cDNA. A cDNA library was constructed from mRNA isolated from adult human liver in the direct expression vector λ gt11 essentially as described.²⁴⁻²⁸ Poly(A)⁺ RNA was isolated and cDNA synthesized essentially as described by Buell et al.²⁴ and Wicken et al.²⁵ The cDNA was made blunt ended by incubation with S1 nuclease²⁶ and the Klenow fragment of DNA polymerase I.²⁷ The cDNA was then methylated with *Eco*RI methylase followed by ligation of *Eco*RI linkers and *Eco*RI digestion. After size fractionation to yield cDNA greater than 1 kb, the cDNA was ligated into *Eco*RI digested and phosphatase-treated λ gt11.²⁸ Approximately 300,000 λ gt11 recombinant phage plaques on *E. coli* Y 1090²⁸ were screened with the IgG fraction of rabbit polyclonal antibody against factor X² and antibody reactions were detected by using horseradish peroxidase-labeled second antibody. After purification, the isolates were screened with a mixture of five factor X-specific mouse MoAbs.²⁹ An independently isolated plaque containing a recombinant phage whose fusion polypeptide reacted with rabbit anti-human ferritin (M. Jaye, unpublished data) was used as control. The cDNA

insert was excised using a combination of restriction enzymes *Eco*RI and *Pvu*II and subcloned into these sites in pBR322. (The recombinant plasmid was referred to as pDH-1.) The cDNA insert was also subcloned between *Eco*RI and *Hinc*II sites of M13mp11. The recombinant phage was isolated and subjected to nucleic acid sequence analysis as described.³⁰ Isolation and characterization of the actin cDNA clone has been previously reported.²²

Isolation of RNA. For RNA isolation sterile disposable plasticware and, when needed, dry heat sterilized glassware were used. When appropriate, stock solutions were treated with 0.1% diethylpyrocarbonate and autoclaved. For Northern blot analysis RNA from tissues and large volume tissue culture was isolated by two methods. Method I was a modification of guanidinium isothiocyanate method,³¹ and method II involved centrifugation through CsTFA. For both methods cells were washed in ice cold 0.15 mol/L NaCl + 1.5 mmol/L HBI and placed on ice. The cells were harvested in 25 mmol/L sodium citrate, pH = 7.0, 0.5% Sarkosyl, 0.1 mol/L 2-mercaptoethanol, 5.6 mol/L guanidinium isothiocyanate. Approximately 15 mL of this solution was used for 10 T-175 flasks. From this point Method I was essentially as described.³¹

Method II involved layering 7 to 8 mL of the guanidinium isothiocyanate lysate of the cells on top of 3 mL of CsTFA ($d = 1.6$ g/mL) in 0.1 mol/L Tris-HCl, 0.1 mol/L KCl, 1 mmol/L EDTA (pH 8.0) in Beckman 16 $\times$ 76 mm Quick-seal centrifuge tubes. After centrifugation for 24 hours in a Ti-50 rotor at $100,000 \times g$, the supernatants were removed, and the pellets and sides of the tube were washed with 3 mL of 6 mol/L guanidine-HCl, 25 mmol/L sodium citrate, and 10 mmol/L 2-mercaptoethanol (pH 7.0). Approximately four pellets were dissolved in 3 mL of this solution and transferred to a clean tube, in which the RNA was precipitated with 2.2 volumes of cold 100% ethanol. The precipitate was washed twice with 70% ethanol, dissolved in RNase-free H₂O, and stored at -70°C . Polyadenylated mRNA was separated from other RNA species by binding to oligo(dT)-cellulose as described.³²

For slot-blot analysis, culture medium was removed from the cells, and the cells were harvested with 3.0 mL of 0.05% (w/v) trypsin and 0.5 mmol/L EDTA in MEM media. The trypsin digestion was stopped with 8 mL of ice cold MEM + 10% FCS, and 1 mL samples were removed for whole cell counts. The cells were centrifuged at $1500 \times g$, washed in ice cold 0.15 mol/L NaCl, 1.5 mmol/L HBI, 0.5 mmol/L aurointricarboxylic acid, pH 7.0, and transferred to microcentrifuge tubes. Three hundred microliters of proteinase K buffer (0.1 mol/L Tris-HCl, 10 mmol/L EDTA, 0.15 mol/L NaCl, 1% SDS; pH 7.5) was added, and the DNA from the lysed cells was sheared by passage through a 22-gauge needle. Proteinase K was added (300 μ g/mL) to each tube and incubated at 37°C for 45 minutes. Protein was removed by phenol:chloroform extraction, the organic phase reextracted with 200 μ L of 20 mmol/L Tris-HCl, 2 mmol/L EDTA, 0.1% SDS, pH 7.4. The pooled aqueous phases were extracted with chloroform followed by ethanol precipitation. The precipitated nucleic acids were dissolved in 80 μ L H₂O, made 40 mmol/L Tris-HCl, 10 mmol/L NaCl, 8 mmol/L MgCl₂, pH 7.9 and 10 U (10 μ L) of RNase-free DNase I and incubated 45 minutes at 37°C . DNase I was chloroform extracted, and the RNA concentrated by ethanol precipitation in 0.25 mol/L sodium acetate, pH 5.2. The precipitate was dissolved in RNase-free H₂O and the absorbance at 260 nm and 280 nm measured. If the $A_{260\text{nm}}/A_{280\text{nm}}$ ratio was less than 1.95, the samples were treated with DNase I until the ratio approached 2.0. The extent of the enzymatic digestions was tested by subjecting samples to RNase digestion.

Northern blot analyses. Northern hybridizations were done essentially as described.^{27,33} RNA samples were denatured with 1.0 mol/L glyoxal or 12.5 mmol/L methyl mercury hydroxide and electrophoresed in 1.2% horizontal agarose gels. After electrophoresis, gels were soaked 20 to 30 minutes in 10 mmol/L sodium

phosphate, pH 6.5, and the RNA transferred for 15 to 20 hours to nitrocellulose presoaked in $20 \times$ standard saline citrate (SSC = 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate, pH 7.0). After transfer, the nitrocellulose was removed, air dried, and then baked at 70°C to 80°C in vacuo for two hours. The nitrocellulose was prehybridized 24 hours at 42°C in a solution containing $5 \times$ SSC, 50% formamide, $5 \times$ Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin) and 100 $\mu\text{g}/\text{mL}$ denatured salmon sperm DNA.

The probes for hybridization reactions were made from nick translation of the pBR322 plasmid containing the subcloned factor X or actin cDNA insert.²⁷ The specific radioactivities of the probes varied from 5 to 50×10^7 cpm/ μg . Unincorporated nucleotides were removed by ethanol precipitation in the presence of 0.4 mol/L ammonium acetate, pH 5.2. The precipitated probes were boiled in 40 μL containing 1 mg/mL solution denatured salmon sperm DNA for ten minutes and cooled on ice. The denatured probes were added to a solution of the same composition as the prehybridizing solution plus 0.1% sodium pyrophosphate and 200 $\mu\text{g}/\text{mL}$ polyadenylic acid. The factor X probe was diluted to 1×10^7 cpm/mL and actin probe was diluted to 1×10^6 cpm/mL for hybridization reactions. Approximately 16 μL of probe was used per cm^2 of nitrocellulose paper. Hybridization was performed for 48 hours at 42°C . After incubation, the nitrocellulose was washed for five minutes in $2 \times$ SSC, 0.1% SDS at 65°C and three 30-minute washes in the same solution followed by three 30-minute washes in $0.2 \times$ SSC, 0.1% SDS. The nitrocellulose was air dried, and the hybridization reactions detected by autoradiography.

For slot-blot analysis appropriate concentrations of the RNA samples were made $10 \times$ SSC and denatured in 8% formaldehyde by heating at 65°C for 10 minutes. The samples were cooled to ambient temperature and diluted in $10 \times$ SSC so that the appropriate amount of RNA could be applied in 100 μL aliquots to nitrocellulose paper (0.45 μm) previously wetted in H_2O and presoaked in $20 \times$ SSC. The procedures for baking, hybridization, and washing of the nitrocellulose were as described above. The autoradiographs were quantitated by scanning with a laser densitometer (Zeineh soft laser scanning densitometer, Biomed Instruments, Inc, Chicago).

RESULTS

Four cDNA clones positive for factor X were detected from screening 300,000 plaques of a human liver cDNA expression library with rabbit anti-factor X antibody. After three cycles of plaque purification, one plaque was picked for further analysis. This clone showed a positive reaction when exposed to a mixture of five factor X-specific MoAbs, while the negative control plaques (ferritin cDNA clones) did not. The cDNA insert from this clone was excised with *Eco*R1 and *Pvu*II and found to be approximately 1100 bp long. Fig 1 shows a partial nucleotide sequence from the 5' end of the cDNA along with the deduced amino acid sequence and a previously published amino acid sequence for the light chain

A	TGG	AAT	AAA	TAC	AAA	GAT	20	GAC	CAG	TGT	GAG	ACC	AGT	40	TGC	CAG
	TRP	ASN	LYS	TYR	LYS	ASP	GLY	ASP	GLN	CYS	GLU	THR	SER	PRO	CYS	GLN
	41															
B	TRP	ASN	LYS	TYR	LYS	ASP	GLY	ASP	GLN	CYS	GLU	THR	SER	PRO	CYS	GLN
C	41															

Fig 1. Partial nucleotide sequence of human factor X cDNA (A). The predicted amino acid sequence (B) and the published sequence (C) are also shown beginning with residue 41. Numbers above line A indicate the nucleotide number of the isolated factor X clone, while numbers below line C represent the established amino acid sequence number.

of factor X.³⁴ The nucleotide sequence is identical to that reported earlier¹⁹ and corresponds to amino acid residues 41 to 56 of the light chain of factor X indicating that this cDNA clone represents a bona fide factor X cDNA which includes portions of both the light and heavy chains.

RNA was isolated from tissue using the guanidinium isothiocyanate technique (Method I).³¹ This and centrifugation through CsTFA (Method II) were used to isolate RNA from cells in culture, and both methods gave RNA with $A_{260\text{nm}}/A_{280\text{nm}}$ absorbance ratios nearly 2.0. Agarose gel electrophoresis demonstrated no detectable degradation of 28S and 18S ribosomal RNAs. Poly (A)⁺ RNA isolated from all samples of total RNA behaved similarly in in vitro translation assays and in cDNA synthesis. Nevertheless, controlled experiments showed that Method II yielded at least 5-fold more RNA than Method I ($\sim 116 \mu\text{g}/10^7$ cells v $20 \mu\text{g}/10^7$ cells) for cell culture samples. Presumably the difference in yields results from the single step isolation of Method II compared to multiple ethanol precipitations required in Method I. We found that pelleting RNA through CsTFA instead of isopycnic banding³⁵ of the nucleic acids required shorter centrifugation times and that pellets were easier to recover than by separating banded nucleic acids.

The size and number of mRNA species encoding factor X was examined using Northern blot analyses with RNA from the Hep G2 cell line, from human liver, and from a human breast tumor cell line (BT-20), which does not secrete factor X. Hybridizations of various concentrations of RNA from the Hep G2 cell line with the factor X nick-translated probe demonstrated a single mRNA species of 1700 bases (Fig 2, left panel). Northern blots of Poly (A)⁺ RNA from BT-20 cells as well as total RNA from liver and Hep G2 cells indicated that the factor X probe hybridized with only one mRNA size of 1700 bases in RNA from both liver and Hep G2 cells (Fig 2, right panel). The factor X probe did not hybridize with RNA from the breast tumor cell line, human

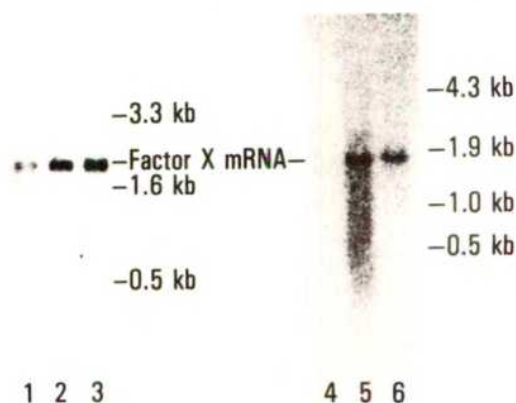


Fig 2. Northern-blot analysis of RNA from human liver and the Hep G2 and BT-20 cell lines. RNA was denatured in either glyoxal (lanes 1 through 3) or methyl mercury hydroxide (lanes 4 through 6) and electrophoresed in 1.2% agarose gels as described^{27,33} and explained in methods. Recombinant plasmid pDH-1, containing a 1.1-kb factor X cDNA insert, was nick-translated and used as the hybridization probe. (Lanes 1 through 3) 5, 7.5, 10 μg of total Hep G2 RNA; (lane 4) 2.5 μg Poly (A)⁺ mRNA from the BT-20 cell line; (lane 5) 7.5 μg liver total RNA; (lane 6) 7.5 μg Hep G2 total RNA.

kidney total RNA, or poly (A)⁺ from a human cervical carcinoma cell line (Me-180) (data not shown) indicating the restriction of factor X gene expression to hepatocytes.

Total RNA for slot-blot analysis was rapidly isolated from numerous small tissue culture samples by Method II. The time from cell harvest to blotting of RNA samples was as rapid as two days with a number of convenient stopping places in the procedure. It was critical to measure the $A_{260\text{nm}}/A_{280\text{nm}}$ ratio after DNase digestion to make sure the enzyme reaction was complete, because DNA contamination interfered with accurate quantitation of mRNA. Samples that were not DNase digested but were subjected to RNase gave strong hybridization signals indicating DNA contamination of the total RNA preparation (data not shown). When samples exhibiting high $A_{260\text{nm}}/A_{280\text{nm}}$ absorbance ratios were subjected to RNase A digestion, no hybridization was observed, indicating that the DNase digestion was complete. Usually 10 units of DNase were required to digest the DNA from approximately 3×10^6 cells and reflected an increase of the $A_{260\text{nm}}/A_{280\text{nm}}$ ratio from around 1.8 to greater than 1.95. Increasing the enzyme concentration or incubation time did not eliminate the need for an occasional second DNase digestion, suggesting that an enzyme inhibitor remaining from previous RNA purification steps is more efficiently removed by a second chloroform extraction. We attempted to use unfractionated cytoplasmic samples developed for dot-blots.³⁶ When it was necessary to blot high concentrations of RNA, we found it difficult to quantitatively transfer the RNA present in the cytoplasmic samples due to the limited surface area of the nitrocellulose membrane in the slot-blot apparatus. We observed that transfer was quantitative up to 8 μg of RNA and that binding was sensitive to RNase.

To determine if the warfarin- and vitamin K-modulated differences in synthesis and secretion of vitamin K-dependent proteins resulted from altered steady state levels of mRNA, we employed quantitative slot-blot analyses. Twelve seven-day-old Hep G2 cultures were washed with serum-free medium and divided into three groups which received either

medium alone or medium containing warfarin or vitamin K. After 24 hours, one flask from each group was removed and processed for cell number determination and RNA isolation. With the nine remaining flasks, subgroups of three flasks that were initially treated with vitamin K, warfarin, or media alone were distributed so that a sample from each group received either the same treatment or one of the other two treatments. After an additional 24 hours of culture, all the flasks were harvested, cell number determined, and RNA isolated. The results from these combinations were then subjected to analyses of variance to determine if any trends developed due to vitamin K or warfarin treatment. Total protein and DNA synthesis in Hep G2 cells are unaffected by warfarin and vitamin K (Bahnak and Fair, unpublished). We used hybridization to an actin cDNA clone and normalization of factor X mRNA to actin mRNA to control for fluctuations in the recovery of internal mRNA populations not resulting from the vitamin K or warfarin treatment.

Representative slot-blot analyses and tracings of the densitometric scans are illustrated in Fig 3 for actin and factor X mRNA after 24 hour treatment with warfarin, vitamin K, or medium alone. The integrated areas under the curves of the densitometer scan were linear through the concentration range of RNA tested, and for quantitative analysis, the tracings for the middle loadings (1.5 and 3 μg mRNA) were used. No changes were detected in the levels of either actin or factor X mRNA with drug treatment relative to the medium only control. This was the case for all pharmacologic combinations in the study. Since there are no reports of the effects of vitamin K or warfarin on the levels of cytoplasmic actin or its mRNA, the ratio of factor X mRNA to actin mRNA would reveal any changes in the relative abundance of factor X mRNA. Table 1 summarizes the results for ratios of factor X mRNA to actin mRNA in Hep G2 cells subjected to the 3×3 fixed factor analysis of variance. Although some cultures showed a higher apparent ratio, the amount of factor X mRNA remained constant for the various groups and subgroups. These values were used for the analysis of

Fig 3. Examples of slot-blot analyses of actin and factor X mRNA from Hep G2 cells treated for 24 hours with vitamin K or warfarin. Total cellular RNA from Hep G2 cells was isolated, serially diluted, and blotted onto nitrocellulose as described in Methods. The pDH-1 (factor X) or an actin cDNA²² recombinant plasmid was nick-translated and used as the hybridization probe. (Lanes 1 and 4) RNA from Hep G2 cells treated with 10 $\mu\text{g}/\text{mL}$ vitamin K; (lanes 2 and 5) RNA from cells treated with 1 $\mu\text{g}/\text{mL}$ warfarin; (lanes 3 and 6) RNA from cells treated with media only. Below the slot-blots are the densitometric tracings for lanes 1 and 4. The numbers below the scans correspond to the amount of RNA in the blot. The integrated readings quantitate the amount of mRNA within the actin (lanes 1 through 3) or factor X (lanes 4 through 6) RNA blots but not between.

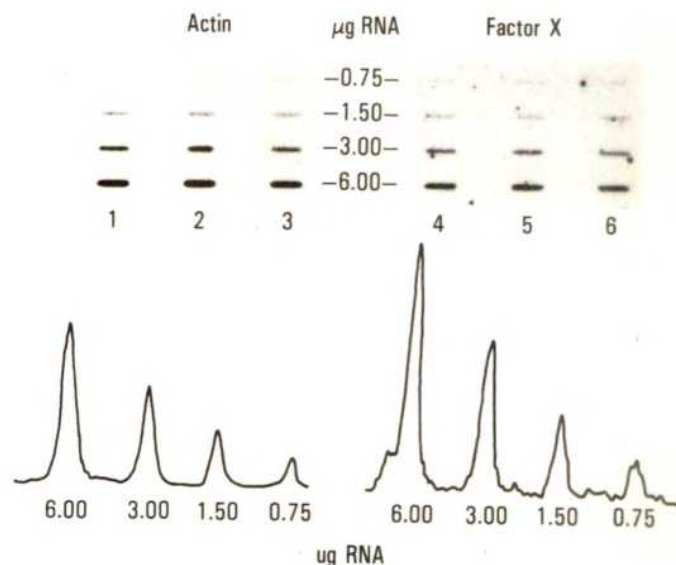


Table 1. Ratios of Factor X mRNA to Actin mRNA in Hep G2 Cells Treated With Vitamin K and Warfarin*

B/A	Vitamin K	Warfarin	Control
Vitamin	0.92	1.27	1.00
Warfarin	1.31	0.94	1.10
Control	1.45	1.13	1.15

*Hep G2 cells were cultured in serum-free medium only (control) or in medium containing 10 µg/mL vitamin K or 1 µg/mL warfarin for 24 hours (A). Subgroups of cells were washed and cultured for an additional 24 hours in vitamin K, warfarin, or control serum-free medium (B). RNA was isolated from each subgroup culture and blotted with radioactive probes made from nick-translated factor X or actin cDNA inserts. Densitometric tracings of the resulting autoradiograms were integrated, and the arbitrary units of integration were used to determine the ratio of factor X mRNA to actin mRNA.

variance for these ratios (Table 2). Table 2 also summarizes the analyses of variance from the individual RNA blots, total RNA, total secreted protein, and cell number. There were no indications of an effect of warfarin or vitamin K on cell number, total RNA, actin mRNA, the ratio of factor X mRNA to actin mRNA, or total secreted protein for any combination of treatment, as all of the F values were <6.94. Thus, the changes in factor X protein secretion observed with warfarin treatment are not the result of modulation of the steady state level of factor X mRNA.

DISCUSSION

Administration of warfarin to patients results in decreased enzymatic activity and antigen levels of factor X and other vitamin K-dependent coagulation proteins.⁴⁻⁹ Changes in the rate of transcription, translation, protein turnover, or protein secretion may account for this observation.¹⁰⁻¹³ We chose to

Table 2. ANOVA Table for Assays on Vitamin K and Warfarin Treated Hep G2 Cells

Assay*	Mean Square	F-ratio†
Cell Number	A‡ 7.8 × 10 ⁹ B‡ 5.3 × 10 ⁹	2.23 1.50
Total RNA	A 4.0 × 10 ⁻⁴ B 2.6 × 10 ⁻²	0.02 1.17
Actin mRNA	A 3.62 B 4.51	1.37 0.94
Factor X mRNA	A 3.0 × 10 ⁻²	0.84
Actin mRNA	B 2.3 × 10 ⁻²	0.63
Total	A 46.7	0.11
Secreted Protein	B 26.0	0.20

*The mean ± standard deviation and (range) of all of these experiments as a whole were: cell number, 1.16 ± 0.23 × 10⁷ (0.75 to 1.44 × 10⁷) cells; total RNA, 91.5 ± 16.5 (62.5 to 126) µg; actin mRNA 13.2 ± 2.4 (11 to 19) integration units; factor X mRNA, 14.5 ± 2.6 (10 to 20) integration units; factor X mRNA/actin mRNA, 1.14 ± 0.18 (0.92 to 1.45); total secreted protein 896 ± 170 (624 to 1116) µg.

†F.95 (2, 4) = 6.94.

‡A = mean square and F-values associated with pretreatments; B = mean square and F-values associated with drug treatments.

examine the effect of warfarin treatment on the steady state levels of factor X mRNA as a first approach to addressing this question. The site of action of this drug is principally on the carboxylating enzyme system present in liver hepatocytes.^{2,3} Hep G2 cells secrete most of the vitamin K-dependent proteins and respond to warfarin and vitamin K in a manner indistinguishable from normal hepatocytes.^{12,21} Therefore, we used this cell line as a model to analyze the mechanism of action for the reduced synthesis of this family of proteins.

A factor X cDNA clone was isolated, partially sequenced, and demonstrated to represent the 5' end of the coding sequence with a nucleotide sequence corresponding to amino acid residues 41 to 56 of the factor X light chain. Our results were identical to those recently reported by another group using nucleotide sequence¹⁹ as well as conventional amino acid sequence analysis.³⁴ This clone was used in northern-blot analyses to identify a single mRNA species of approximately 1700 bases in both the Hep G2 cell line and human liver. Therefore, the mature mRNA in Hep G2 cells and liver appear to be transcribed and processed in the same manner. Since our factor X clone contains nucleotide sequences that encode for both the heavy chain as well as the light chain of factor X, the single mRNA species identified in northern blots further supports the conclusion that human factor X is synthesized as a single chain molecule and subsequently cleaved to the conventionally recognized two-chain form.^{12,18,19}

When the length of mRNA for factor X is compared to sizes of factor X cDNA clones,^{18,19} it appears that about 200 to 250 bases in the mature mRNA may not be translated. The longest reported cDNA clone for factor X is approximately 1440 bp and appears to lack sequences for part of the leader peptide, 5'-untranslated regions and most of the poly (A)⁺ tail.¹⁹ Factor X is expected to have similar leader sequences as other vitamin K-dependent proteins, and although it is difficult to estimate the length of the 5'-untranslated regions, other vitamin K-dependent coagulation factors in bovine and human appear to have a short 5'-untranslated region suggesting that the majority of the untranslated nucleotides in mature factor X mRNA may be in the polyadenylated tail.^{17,19,37-39}

We used factor X and actin recombinant cDNA clones as probes to investigate vitamin K and warfarin treatment on the levels of mRNA in Hep G2 cells. While warfarin specifically reduces secretion of the γ-carboxylated coagulation proteins,^{12,21} the response of these cells to vitamin K is less striking and inconsistent; however, increased levels of prothrombin have been observed.¹² Furthermore, factor X and prothrombin levels respond to vitamin K in cultured rat hepatoma cells.¹⁰⁻¹¹ Measurements of extra- and intracellular levels of factor X and prothrombin indicated that secretion alone could not account for the changes observed in these proteins in response to warfarin or vitamin K.¹⁰⁻¹² Therefore, these drugs may alter the synthetic rates at the transcriptional level of these proteins produced by the hepatoma cells as has been suggested for the in vivo condition.⁴⁰ Among the possibilities for the regulation of mRNA levels might be the stability of the transcripts following drug

treatment.^{41,42} In the present study we found no changes in the levels of total RNA or specific factor X mRNA following incubation of cells with warfarin or vitamin K. Even samples that had duplicate application of the same agent in the experiment showed no alterations in the RNA parameters measured. Thus, it appears that neither warfarin nor vitamin K affect the steady state levels of mRNA in Hep G2 cells. Whether the maintenance of the steady state levels in the drug treated cells is in fact due to an increased rate of transcription of a less stable transcript is unknown; further studies, including measurement of transcript stability, would be necessary to confirm or deny this hypothesis.

Regulation of vitamin K-dependent protein synthesis could occur at the level of translation, intracellular protein turnover, or rate of secretion. Previously, we determined that the changes in the secretion of prothrombin could not be accounted for by increases (warfarin treatment) or decreases (vitamin K addition) in the intracellular concentration of prothrombin.¹² Thus, either changes in the rate of translation or intracellular turnover must be responsible for the protein changes observed in vitro and in vivo in response to these pharmacologic agents. Altered protein conformation due to

covalent modifications, cofactor dissociation, or amino acid substitutions can change the degradation rate.⁴³⁻⁴⁵ The effects of protein carboxylation on turnover rates are not known, but during vitamin K-deficient states the vitamin K-dependent proteins would not be processed normally, and these incompletely modified proteins may be more susceptible to intracellular catabolism. If changes in turnover rates are combined with changes in secretion rates, the effects on extracellular levels of these proteins could be amplified. Changes in both turnover and secretion rates have been suggested to account for intraspecies differences in the amount of descarboxyprothrombin present in the plasma of warfarin treated animals.^{46,47} Since the carboxylase system in Hep G2 cells appears to respond "normally" to vitamin K and warfarin, it should provide a system to analyze the effects of γ -carboxylation on the turnover of this family of homologous coagulation proteins.

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Protein S Is a Cofactor for Activated Protein C Neutralization of an Inhibitor of Plasminogen Activation Released From Platelets

By Armando D'Angelo, Marion S. Lockhart, Silvana Vigano D'Angelo, and Fletcher B. Taylor, Jr.

Platelets stimulated with thrombin release an inhibitor of plasminogen activator (PAI), which has been shown previously to be neutralized by activated protein C (APC). The requirements for optimal neutralization of PAI activity were investigated. The releasate of gel-filtered human platelets stimulated with thrombin served as a source of PAI. When 6×10^8 platelets/mL were incubated with thrombin (1 IU/mL), the releasate contained 18 to 26 ng/mL PAI as determined by incubation of the releasate with urokinase and measurement of residual urokinase activity on plasminogen (S2251). Preincubation of PAI with up to 4 μ g/mL APC for two hours yielded less than 20% neutralization of PAI activity. In the presence of protein S, phospholipid, and Ca^{2+} , neutralization of PAI activity was

time-dependent with 50% neutralization occurring in two hours with 1 μ g/mL APC. The cofactor effects of protein S and phospholipid were concentration-dependent with half-maximal acceleration at approximately 3 μ g/mL protein S and 10 μ g/mL phospholipid when the experiments were performed at 1 μ g/mL APC. Diisopropylfluorophosphate-inactivated APC, gla-domainless APC, and thrombin-cleaved protein S had no effect on PAI activity, indicating requirement for preservation of the APC active site and of the Ca^{2+} binding ability of both APC and protein S. These results suggest coordinate binding of APC and protein S onto phospholipid membrane as a prerequisite for optimal expression of PAI neutralized by APC.

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ACTIVATED PROTEIN C accelerates whole blood clot lysis *in vitro*¹ by multiple mechanisms, many dependent on or accelerated by the presence of mononuclear leukocytes and platelets.² One mechanism by which activated protein C produces this effect is by neutralizing an inhibitor of plasminogen activator released from mononuclear leukocyte-platelet mixtures.³ Activated protein C also neutralizes a plasminogen activator inhibitor found in endothelial cell conditioned media.^{4,5} Immunologic identity of the platelet- and endothelial cell-derived plasminogen activator inhibitors (PAI) had been previously demonstrated.⁶ It was suggested that activated protein C prevented inhibition of tissue plasminogen activator by preventing complex formation with PAI.^{7,8} However, the concentrations of activated protein C required to produce these effects were high relative to the other constituents and to the amount of activated protein C one might expect to be produced under physiologic conditions.

Since protein S is a cofactor for the hydrolysis of factor Va and the anticoagulant activity of activated protein C,⁹ we asked if protein S might also be a cofactor for neutralization of plasminogen activator inhibitor and thus facilitate profibrinolytic activity of activated protein C.

Therefore, we studied the conditions and components necessary for optimum activated protein C neutralization of the PAI released from platelets by thrombin.

MATERIALS AND METHODS

Fibrinolytic Components and Assay

Preparation of fibrinolytic components. Human glu-plasminogen (specific activity of 18 to 20 CU/mg) was purified as described¹⁰ and stored in 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.55 at -60°C .

S-2251 (Kabi Diagnostica, Stockholm, Sweden) was stored in 0.5 mol/L NaCl at 3.6 mmol/L concentrations in 1 mL aliquots at -20°C .

High molecular weight human urokinase, (100,000 IU/mg; Calbiochem Corp., La Jolla, Calif) was stored in 50% glycerol, 20

mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.55, at -20°C at a concentration of 1000 U/mL.

Human plasmin (15 CU/mg, Kabi Diagnostica, Stockholm, Sweden) was stored in 50% glycerol, 0.001 N HCl, at -20°C at a concentration of 0.5 mg/mL.

Assay and characteristics of fibrinolytic components. Activation of plasminogen to plasmin by urokinase was monitored using S-2251 as substrate.¹¹ This assay was run in three stages to accommodate the requirements of the full-scale experiments in which all components were used. In stage 1, platelet releasate (source of PAI) was incubated for 60 to 240 minutes at 37°C either with Tangen HEPES-0.1% BSA buffer,¹² or with one or more of the following: activated protein C, diisopropylfluorophosphate-inactivated activated protein C, gla-domainless activated protein C, bovine factor Xa, protein S, thrombin-cleaved protein S, phospholipid, CaCl_2 , or EDTA in a final volume of 0.11 mL.

In stage 2, urokinase (10 μ L) was added and incubated for ten minutes at 37°C . For each experiment a calibration curve was constructed with increasing urokinase concentrations (0.5 to 5.0 IU/mL) incubated with 0.11 mL buffer (final volume 0.12 mL). Urokinase activity was stable in buffer for up to six hours at 37°C . In stage 3, 40 μ L of this stage 2 incubate was added to 30 μ L of plasminogen giving a final concentration of 70 μ g/mL of plasminogen. After incubation of this mixture for 15 minutes at 37°C , 30 μ L of S-2251 was added giving a final concentration of 1.0 mmol/L S-2251. The total volume of stage 3 was 0.1 mL. The reaction was stopped by addition of 0.5 mL of 1% acetic acid when the color intensity appeared such that it would have an absorbance of 0.600 (8 to 40 minutes). The extent of hydrolysis of S-2251 by plasmin was

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measured by determining absorbance at 405 nm using a Beckman DU7 spectrophotometer.

Under these conditions, kinetic analysis of the rate of S-2251 hydrolysis during stage 3 showed a linear increase of $\Delta A_{405}/\text{min}$ over time for urokinase concentrations in stage 2 ranging from 0.25 to 3 IU/mL, provided sufficient S-2251 was available. This suggested that although plasminogen activation was still taking place during stage 3, this occurred as a linear function of the urokinase concentration present at completion of stage 2. Fig 1 shows a typical concentration-dependent activation of plasminogen by urokinase incubated with either buffer or platelet releasate.

It should be noted that since the reaction of greatest interest involving neutralization of PAI activity in platelet releasate occurred in stage 1 of this assay, the concentration of platelet releasate (% dilution), activated protein C, diisopropylfluorophosphate-inactivated activated protein C, gla-domainless activated protein C, bovine factor Xa, protein S, thrombin-cleaved protein S ($\mu\text{g/mL}$), phospholipid ($\mu\text{g/mL}$), CaCl_2 (mmol/L), and EDTA (mmol/L) are given with respect to stage 1.

Antifibrinolytic Components and Assay

Preparation of platelet releasate. Human platelets (1 to $5 \times 10^9/\text{mL}$), prepared from platelet concentrates within two to four hours of collection in ACD buffer, were incubated with 0.1 mg/mL prostaglandin E_1 at 22°C . After 10 minutes they were gel-filtered through Sepharose 2B equilibrated in Tangen HEPES- 0.1% BSA buffer as described by Carroll et al.¹³

Platelet releasate was prepared from 5 to 7 mL of washed platelets after incubation for 30 minutes at 37°C . The platelets were aggregated by addition of thrombin (1 IU/mL) and centrifuged at $2,000 \times g$ for ten minutes at room temperature. The supernate was harvested and gentamicin (0.25 mg/mL) and (p-amidinophenyl) methanesulfonyl fluoride ($20 \text{ }\mu\text{mol/L}$) were added to inhibit bacterial growth and residual thrombin, respectively.

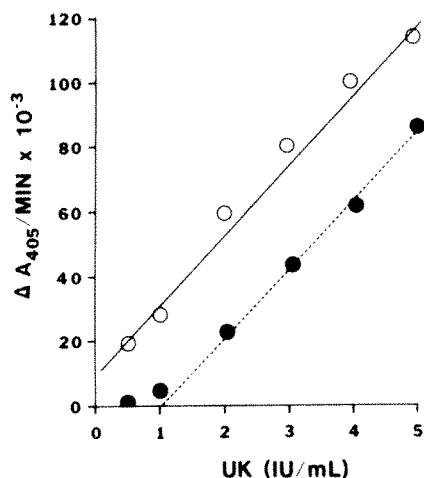


Fig 1. Inhibition of urokinase activation of plasminogen by PAI in platelet releasate. Stage 1: Tangen HEPES- 0.1% BSA buffer ($\circ$ - $\circ$) or a 50% dilution of platelet releasate in buffer ($\bullet$ - $\bullet$) were incubated for two hours at 37°C (final volume 0.11 mL). Stage 2: Urokinase (0.01 mL) was added to give the final concentrations as shown and incubated for ten minutes at 37°C . Residual urokinase activity on plasminogen was followed in stage 3 as described in Methods. The intercept of the platelet releasate-urokinase concentration dependence curve allows calculations of PAI activity in the platelet releasate. This preparation of platelet releasate (undiluted) contained approximately 20 ng/mL active PAI.

Platelets release protein S upon stimulation with thrombin.^{14,15} According to Schwarz et al¹⁴ between 0.2 and $0.4 \text{ }\mu\text{g}$ protein S is recovered in the supernatant of 3×10^8 platelets stimulated with 1 IU/mL thrombin. To remove protein S from platelet releasate, a mouse monoclonal antibody directed against human protein S (HPS-2, a gift from Dr C.T. Esmon) was used. This was coupled to Affigel 10 (Biorad, Richmond, Calif) at a concentration of 5 mg/mL gel and 0.2 mL of packed beads was incubated with 5 mL of platelet releasate for two hours at room temperature on a rocker. The immobilized antibody was removed by centrifugation. Under the same conditions, up to $50 \text{ }\mu\text{g/mL}$ purified protein S can be completely removed by the immobilized monoclonal antibody as judged by immunoelectrophoresis. Once stored at 4°C , PAI activity remained stable for two to three weeks in the platelet releasate.

Assay and characterization of PAI activity of platelet releasate. A 50% dilution of stock platelet releasate was used in this and all the other studies that follow. This stock contained releasate generated from $1.2 \times 10^9/\text{mL}$ platelet suspensions. Fig 1 shows that the PAI activity of platelet releasate followed a linear pattern in parallel with the urokinase control. Since under these conditions the reaction of PAI with urokinase was complete at all concentrations within the ten-minute incubation period, we concluded that the reaction was stoichiometric as previously suggested.⁶ The intercept of the platelet-releasate-urokinase concentration dependence curve can be used to estimate the amount of active PAI in platelet releasate. In Fig 1, a 50% dilution of platelet releasate neutralized completely the apparent activity of at least 1 IU/mL ($=10 \text{ ng/mL}$) urokinase, giving 100% inhibition (closed circles). Assuming a stoichiometric, essentially irreversible interaction between two proteins of approximate equal molecular weight ($53,000$ v $50,000$), 10 ng/mL urokinase would bind an equal weight/mL of PAI.

As a result, a good approximation of the amount of active PAI in platelet releasate (ng/mL) can be derived from measurement of residual urokinase activity on the calibration curve according to the equation: $[\text{UK}]_{\text{added}} - [\text{UK}]_{\text{residual}} = [\text{Active PAI}]$. In spite of the addition of (p-amidinophenyl) methanesulfonyl fluoride, PAI activity in the platelet releasate declined spontaneously at 37°C (apparent half-life 120 to 180 minutes), consistent with the report of spontaneous decline of PAI activity in endothelial-cell-conditioned media.⁴ Therefore, unless indicated otherwise, PAI activity of platelet releasate is always referred to as the apparent activity observed after a two-hour incubation in stage 1.

Control studies in which plasmin (10 to 100 ng/mL) was added to platelet releasate also were done. No inhibition of plasmin by platelet releasate was observed.

Activated Protein C Components

Human protein C was partially purified from plasma (8 to 10 L) as previously described¹⁵ and separated from contaminating proteins by chromatography on a murine anti-human protein C monoclonal antibody (HPC-4) which requires Ca^{2+} for interaction with protein C.¹⁶ Human activated protein C was prepared from protein C daily by means of insolubilized thrombin-thrombomodulin as described previously.¹⁷ The anticoagulant activity of all preparations of activated protein C was assayed using a factor Xa one-stage assay.¹⁷ The stock solutions of $50 \text{ }\mu\text{g/mL}$ activated protein C in 0.02 mol/L Tris-HCl, 0.1 mol/L NaCl, 4 mmol/L CaCl_2 and 0.1% BSA were stored at 4°C no longer than two days. Working solutions of activated protein C were prepared just before each experiment to the final concentration required in Tangen HEPES- 0.1% BSA buffer, pH 7.4. The gla-domain (residues 1 to 44 of the light chain of protein C) was removed by limited proteolysis with chymotrypsin and chromatography as described by Esmon et al.¹⁸ After activation with thrombin-thrombomodulin¹⁷ the gla-domainless activated protein C

had greater than 90% of the amidolytic activity of a corresponding concentration of activated protein C but less than 10% of its anticoagulant activity. Human protein S was prepared as described by Comp et al.¹⁹ Thrombin cleaved protein S was prepared by incubating protein S (0.5 mg/mL) in 1.5 mL of 20 mmol/L Tris-HCl, 2 mmol/L EDTA buffer, pH 7.4, containing 0.1 mol/L NaCl and bovine thrombin at a final concentration of 50 μ g/mL for 15 minutes at 37 °C. The thrombin then was removed by batch adsorption onto 0.3 mL SP-Sephadex for 30 minutes.

Phospholipid suspensions containing 7.5 mg phosphatidylcholine-phosphatidylserine (70:30 PC:PS) per mL 0.015 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.4, were prepared by sonication (Branson model B-3 bath) for 30 minutes at room temperature. These suspensions were stored at room temperature under nitrogen for up to one week.

Other Reagents

The diisopropylfluorophosphate-inactivated activated protein C was prepared by treating activated protein C (0.5 mg/mL) with 10 mmol/L diisopropylfluorophosphate in 0.1 mol/L Tris saline buffer for ten minutes at room temperature. This preparation was dialyzed overnight against 4 L of 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.55, and assayed for activated protein C activity using the factor Xa one-stage clotting assay. Less than one percent of the original activity remained.

A stock solution of human α -thrombin prepared as described by Owen et al.²⁰ 610 NIH U/mL, had specific activities from 2,800 to 3,000 IU/mg and was stored in 5 mmol/L MES, 0.15 mol/L NaCl, pH 6.0, at -60 °C.

Activated bovine factor X (Xa) was prepared from purified factor X as described by Skogen et al.²¹ A stock solution of Xa, 0.5 mg/mL, was stored in 2 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5, at -80 °C. This was a gift from Dr C.T. Esmon.

Bovine serum albumin (BSA) 96% to 99% (fraction V), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), gentamicin, phosphatidylcholine, phosphatidylserine, prostaglandin E₁, Sepharose 2B, QAE-Sephadex, and SP-Sephadex were obtained from Sigma Chemical Co (St. Louis, Mo). The (p-amidinophenyl)methanesulfonyl fluoride and diisopropylfluorophosphate were obtained from Calbiochem Corp (La Jolla, Calif). All chemicals were reagent grade.

RESULTS

The concentration-dependent neutralization of PAI activity in platelet releasate by activated protein C is shown in Fig 2. In the presence of EDTA, activated protein C had no PAI neutralizing activity. Addition of Ca²⁺ and low levels of phospholipid did not produce full expression of the PAI neutralizing activity of activated protein C, unless protein S also was present. Requirement for preservation of the active site of activated protein C was demonstrated by failure of diisopropylfluorophosphate-inactivated activated protein C to produce neutralization of platelet PAI (Fig 2). This closely resembled the conditions for the expression of activated protein C anticoagulant activity, suggesting cofactor-dependent binding of activated protein C to phospholipid as an explanation for its increased efficacy. Requirement for preservation of the ability of activated protein C to interact with phospholipid surfaces was demonstrated by substitution of gla-domainless activated protein C for activated protein C. Gla-domainless protein C is normally activated by soluble

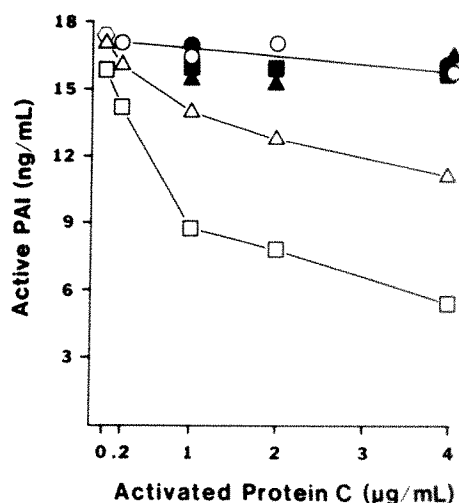


Fig 2. Concentration dependence of activated protein C-induced neutralization of PAI activity in platelet releasate. Stage 1: To a 50% dilution of platelet releasate in Tangen HEPES-0.1% BSA buffer final concentrations of activated protein C as shown were added in the presence of either 3 mmol/L EDTA (○-○), 2 mmol/L CaCl₂ and 10 μ g/mL phospholipid (70:30 PC:PS) with (□-□) or without (△-△) 10 μ g/mL protein S (final volume 0.11 mL). Platelet releasate (50% dilution) was also incubated with either buffer (○), bovine Xa (1,2,4 μ g/mL, ▲-▲), gla-domainless activated protein C (1,2,4 μ g/mL) in the presence of 2 mmol/L CaCl₂, 10 μ g/mL protein S, and 10 mg/mL phospholipid (■-■), or activated protein C at the shown concentrations with a 10 molar excess of diisopropylfluorophosphate-inactivated activated protein C in the presence of 10 μ g/mL phospholipid, 2 mmol/L CaCl₂, and 10 μ g/mL protein S (●-●). These mixtures were incubated two hours at 37 °C. Stage 2: Urokinase then was added to give a final stage 2 concentration of 2 IU/mL, and this mixture was incubated an additional ten minutes at 37 °C. In stage 3, residual urokinase activity was measured on a calibration curve obtained with increasing concentrations of urokinase in buffer (0.5 to 5 IU/mL) and relative ng/mL active PAI calculated as described in Methods.

thrombin-thrombomodulin complex, but has no anticoagulant activity due to impaired binding to phospholipid.¹⁸ Gla-domainless activated protein C, in the presence or absence of Ca²⁺, phospholipid, and protein S, produced no neutralization of PAI activity over the same range of concentrations used for activated protein C (Fig 2).

The specificity of activated protein C in neutralizing PAI activity was suggested by substituting bovine Xa, in the presence of phospholipid and Ca²⁺, for activated protein C. Bovine Xa has similar coagulant activity to human Xa in human plasma.¹⁷ Bovine Xa (1 to 4 μ g/mL) had no PAI neutralizing activity.

Figure 3 shows the time-dependence at 37 °C of the neutralization of PAI activity in the platelet releasate by activated protein C. In this experiment, PAI activity declined spontaneously to 50% in four hours. After two hours, PAI activity was specifically reduced to 41% by 1 μ g/mL activated protein C and to 29% by 4 μ g/mL activated protein C in the presence of protein S, phospholipid, and Ca²⁺. By removal of Ca²⁺ from the incubation mixture with 3 mmol/L EDTA, 4 μ g/mL activated protein C produced only 13% neutralization of PAI activity after two hours.

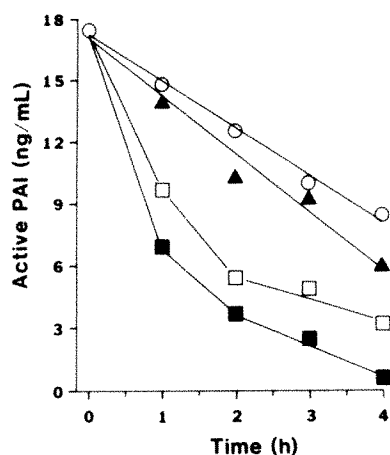


Fig 3. Time course of activated protein C-dependent neutralization of PAI in platelet releasate in the presence of protein S, phospholipid, and Ca^{2+} . Final concentrations of 1 $\mu\text{g/mL}$ activated protein C ($\square$ - $\square$) and 4 $\mu\text{g/mL}$ ($\blacksquare$ - $\blacksquare$) with 10 $\mu\text{g/mL}$ protein S, 10 $\mu\text{g/mL}$ phospholipid (70:30 PC:PS), and 2 mmol/L CaCl_2 or 4 $\mu\text{g/mL}$ activated protein C with the same concentrations of phospholipid and protein S, but with 3 mmol/L EDTA ($\blacktriangle$ - $\blacktriangle$) were added to a 50% dilution of platelet releasate in Tangen HEPES-0.1% BSA in stage 1 at 37 °C. Platelet releasate (50% dilution) was incubated with buffer only, at 37 °C ($\circ$ - $\circ$). At the indicated times urokinase (2 IU/mL) was added and this stage 2 mixture (0.12 mL) incubated for ten minutes at 37 °C. Residual PAI activity (mg/mL) was calculated as described in Methods.

The reconstituted system (activated protein C plus protein S, phospholipid and Ca^{2+}) did not reverse inhibition of urokinase activity by the platelet PAI (Fig 4). Incubation of platelet releasate with urokinase for ten minutes in stage 2, by omitting stage 1, resulted in complete neutralization of 2.5 IU/mL urokinase (line F). Preincubation of platelet releasate with buffer for two hours at 37 °C (stage 1) followed by addition of urokinase produced neutralization of 1 IU/mL urokinase, as a result of spontaneous decline in activity (line D). Activated protein C (4 $\mu\text{g/mL}$) with its cofactors produced virtually complete neutralization of PAI activity after a two hour incubation at 37 °C with platelet releasate in stage 1 (line C). In contrast, incubation of activated protein C with its cofactors, platelet releasate and urokinase for two hours in stage 1 (line E) resulted in a PAI activity which was similar to that of the platelet releasate incubated for ten minutes with urokinase after omission of stage 1 (line F). Urokinase activity was identical upon incubation with buffer for either ten minutes or two hours at 37 °C (lines A and B). These data confirm fast neutralization of urokinase activity by PAI and suggests no significant effect of activated protein C and its cofactors on the equilibrium between urokinase and the PAI.

Neutralization of factor Va by activated protein C is dependent on the concentration of both protein S and phospholipid.²² This appears also to be the case for activated-protein C-dependent neutralization of the platelet PAI. Fig 5 shows the concentration dependence of the cofactor effect of protein S. Increasing concentrations of protein S in the presence of 1 $\mu\text{g/mL}$ activated protein C, phospholipid, and Ca^{2+} reduced PAI activity to 37% with approximate half

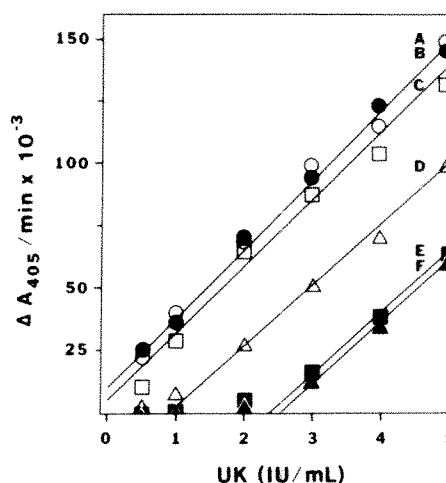


Fig 4. Effect of the presence of urokinase on the neutralization of platelet PAI by activated protein C, protein S, phospholipid and Ca^{2+} . The assay was conducted as described in Methods. Stage 1: All samples described below were incubated for 120 minutes at 37 °C; incubation volumes equal to 0.11 mL. A ($\circ$): buffer (Tangen HEPES-0.1% BSA, pH 7.4); B ($\bullet$): urokinase in buffer; C ($\square$): 4 $\mu\text{g/mL}$ activated protein C (66 nmol/L), 10 $\mu\text{g/mL}$ protein S (147 nmol/L), 10 $\mu\text{g/mL}$ phospholipid, and 2 mmol/L CaCl_2 with a 50% dilution of platelet releasate in buffer; D (Δ): platelet releasate (50% dilution) in buffer; E ($\blacksquare$): urokinase, 4 $\mu\text{g/mL}$ activated protein C, 10 $\mu\text{g/mL}$ protein S, 10 $\mu\text{g/mL}$ phospholipid and 2 mmol/L CaCl_2 with a 50% dilution of platelet releasate in buffer; F ($\blacktriangle$): platelet releasate (50% dilution) with urokinase in buffer. Stage 2: Ten-minute incubation at 37 °C; incubation volumes equal to 0.12 mL; addition of 10 μL of either buffer (B, E, F) or urokinase (A, C, D). All urokinase added to give a final concentration in stage 2 as shown on the abscissa. Stage 3 was followed as described in the text. The platelet PAI was spontaneously reduced to about 50% after incubation at 37 °C for 120 minutes (D, F). Incubation of activated protein C with the platelet releasate for 120 minutes followed by addition of urokinase caused virtually complete neutralization of antiactivator activity (C). In the presence of urokinase activated protein C had no effect on the apparent anti-PAI activity (E, F).

maximal acceleration at 3 $\mu\text{g/mL}$. In the absence of activated protein C, 16 $\mu\text{g/mL}$ protein S with phospholipid and Ca^{2+} produced no neutralization of PAI activity. Addition of 21 $\mu\text{g/mL}$ of goat polyclonal anti-human protein S antibody (kindly donated by Dr P.C. Comp) in stage 1, together with activated protein C, phospholipid, Ca^{2+} , and 10 $\mu\text{g/mL}$ protein S, completely abolished PAI neutralization (not shown). Requirement for preservation of the Ca^{2+} binding ability of protein S to support activated protein C-dependent neutralization of PAI was demonstrated by substituting thrombin-cleaved protein S, which binds Ca^{2+} less effectively and which is devoid of any anticoagulant activity,²³ for native protein S. Thrombin-cleaved protein S showed no cofactor activity (Fig 5). Moreover, removal of Ca^{2+} from the incubation mixture with EDTA completely inhibited PAI neutralization by activated protein C in the presence of protein S and phospholipid (not shown).

The concentration dependence of the cofactor effect of phospholipid is shown in Fig 6. Activated protein C (1 mg/mL) was incubated with recalcified platelet releasate and increasing concentrations of phospholipid for two hours

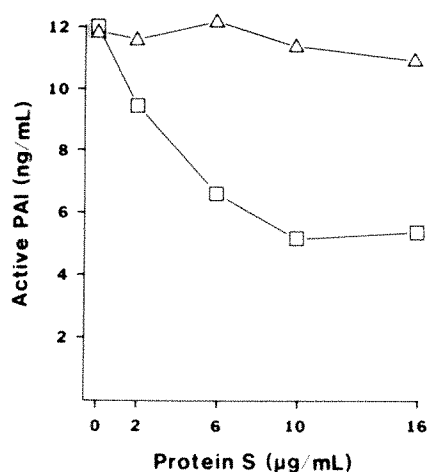


Fig 5. Concentration dependence of protein S on the activated protein C induced neutralization of PAI activity in platelet releasate. Stage 1: To a 50% dilution of platelet releasate in Tangen HEPES-0.1% BSA buffer, final concentration, as shown, of protein S (□-□) or thrombin cleaved protein S (Δ-Δ) were added to 10 μg/mL phospholipid (70:30 PC:PS), 1 μg/mL activated protein C, and 2 mmol/L CaCl_2 . Platelet releasate (50% dilution) was also incubated with buffer. These mixtures (0.11 mL) were incubated two hrs at 37 °C. In stage 2, urokinase was added to give a final stage 2 concentration of 2 IU/mL. Residual active PAI (ng/mL) was calculated as described in Methods. PAI activity was 14 ng/mL in the platelet releasate incubated with buffer (50% dilution) in stage 1. Controls that showed no neutralization of PAI included: protein S plus phospholipid and activated protein C in 3 mmol/L EDTA; protein S plus phospholipid and calcium; protein S alone.

at 37 °C in the presence or in the absence of protein S. Half maximal acceleration of PAI neutralization by activated protein C was observed at approximately 10 μg/mL phospholipid in the presence of protein S as opposed to approximately 30 μg/mL phospholipid in the absence of protein S. At 40 μg/mL and 80 μg/mL phospholipid concentration, protein S cofactor activity was no longer apparent. PAI neutralization was not observed either in the absence of Ca^{2+} or in the absence of activated protein C (with 80 μg/mL phospholipid, Fig 6). If the cofactor effects of protein S and phospholipid are due to interaction of activated protein C with protein S onto the phospholipid surface, one would expect diisopropylfluorophosphate-inactivated activated protein C to behave like a competitive inhibitor of activated protein C. As shown in Fig 2, incubation of a 10 molar excess of diisopropylfluorophosphate-inactivated protein C with increasing concentrations of activated protein C in the presence of its cofactors completely inhibited activated-protein C-dependent neutralization of PAI in platelet releasate.

DISCUSSION

These studies confirm that activated protein C neutralizes the inhibitor of plasminogen activator derived from platelets.³ These studies are the first to demonstrate that protein S is a cofactor for this profibrinolytic effect of activated protein C.

Walker has shown that the rate of factor Va inactivation is

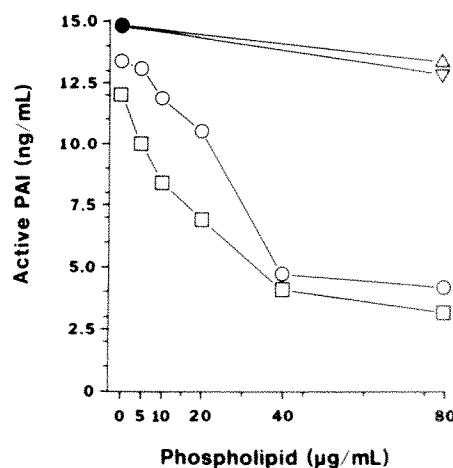


Fig 6. Concentration dependence of phospholipid on the activated protein C induced neutralization of PAI activity in platelet releasate. Stage 1: To a 50% dilution of platelet releasate in Tangen HEPES-0.1% BSA buffer containing 1 μg/mL activated protein C and 2 mmol/L Ca^{2+} , final concentrations of phospholipid (70:30 PC:PS) as shown were added either in the presence (□-□) or in the absence (○-○) of 10 μg/mL protein S. Platelet releasate (50% dilution) was also incubated with buffer (●). These mixtures (0.11 mL) were incubated two hours at 37 °C. In stage 2, urokinase was added to give a final concentration of 2 IU/mL. Residual PAI activity was determined as described in Methods. Controls that showed no significant neutralization of PAI included platelet releasate plus 80 μg/mL phospholipid (Δ), and platelet releasate plus phospholipid (80 μg/mL), 3 mmol/L EDTA, protein S (10 μg/mL), and activated protein C (1 μg/mL) (▽).

increased about eightfold by the addition of protein S to activated protein C.⁹ He also observed that the presence of phospholipid was required for expression of the protein S cofactor activity. He has proposed that protein S increases Ca^{2+} -dependent binding of activated protein C to phospholipid membranes by formation of a 1:1 stoichiometric complex, thus accelerating proteolytic degradation of factor Va on the phospholipid surface.²² Studies on endothelial cell²⁴ and platelet¹⁵ receptors for activated protein C and protein S have confirmed formation of activated protein C-protein S complexes as a prerequisite for factor Va neutralization, although indicating different stoichiometries (1 activated protein C molecule: 20 protein S molecules on platelets; 1:400 on endothelial cells).

Protein S is also required for full expression of the PAI activity of activated protein C. The rate of PAI neutralization is increased at least six- to eight-fold by addition of protein S (Fig 6). Protein S stimulation of PAI inactivation occurred only in the presence of both phospholipid and Ca^{2+} . Both activated protein C and protein S must retain their ability to bind phospholipid vesicles for effective PAI neutralization as shown by the experiments conducted with gla-domainless protein C and with thrombin-cleaved protein S. Therefore, taken together, these data suggest that, as in the case of factor Va neutralization, the qualitative biochemical basis for the cofactor effect of protein S in supporting activated protein C-dependent neutralization of PAI resides in its ability to increase the affinity of activated protein C for phospholipid surfaces. This is consistent with (a) saturability

of both phospholipid and protein S effects (Figs 2 and 6), (b) the finding of no significant protein S cofactor activity in the presence of a large excess of phospholipid (Fig 5), and (c) the inhibitory effect of diisopropylfluorophosphate-inactivated activated protein C on activated protein C (Fig 2).

Accurate quantitative analysis of the concentration dependence of the effects of protein S and phospholipid in our system was hindered by the unavailability of sufficiently high concentration of platelet PAI. Interference by other platelet release products (factor Va, platelet proteases) and/or by residual platelet particles cannot be excluded. Moreover, the estimate of PAI concentration being based on PAI activity does not take into account the possible contribution of inactive forms of PAI. With these limitations, however, we estimate that 44 nmol/L protein S was required for the expression of maximal neutralizing activity of 15.5 nmol/L activated protein C in the presence of 10 μ g/mL phospholipid (Fig 5). When considering that some degree of protein S cleavage commonly occurs during the purification procedures (20% to 30%) and that unknown platelet release products may interfere,²⁵ these figures should be an overestimation of protein S in the stoichiometry of the functional protein S-activated protein C complex on the phospholipid surface.

A major observation is that the concentrations of activated protein C and protein S active in our system are in the physiological range. Approximately 0.10 nmol/L active platelet PAI was specifically neutralized by 15.5 nmol/L activated protein C and 147 nmol/L protein S over a period

of 1 hour (Fig 3). In a whole blood clot lysis system de Fouw et al²⁶ induced half maximal clot lysis in one hour at activated protein C concentrations of 15.5 nmol/L, provided endogenous protein S was present. If their results are due to neutralization of PAI released by physiologic platelet concentrations, this would provide evidence for a physiologic role of the anti-PAI activity of activated protein C. In vivo, sustained generation of activated protein C might thus contribute to the regulation of PAI activity. The clinical consequence of this is that patients with deficiencies of protein C and/or protein S might respond subnormally to a prothrombotic stimulus both during the phase of thrombin formation and fibrin dissolution.

The biochemical mechanism of PAI neutralization by activated protein C remains to be elucidated. Complex formation between the enzyme and the PAI has been reported by Sakata et al,⁵ but not confirmed by van Hinsbergh et al,⁴ who suggested proteolytic cleavage of the PAI as an alternative explanation. Our results confirm that the active site of activated protein C is needed for neutralization of PAI activity and suggest no significant effect of the enzyme plus or minus its cofactors on the equilibrium of the urokinase-PAI complex (Fig 4). Although the similarity observed in the requirements of the PAI neutralizing system and the factor Va neutralizing system are very suggestive of a catalytic action of activated protein C on PAI, only studies with entirely purified components will allow final conclusions.

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Characterization of a Monoclonal Antibody Having Selective Reactivity With Normal and Neoplastic Plasma Cells

By Alex W. Tong, Jennifer C. Lee, and Marvin J. Stone

A myeloma cell-reactive monoclonal antibody (MoAb), MM4, was generated from BALB/c mice immunized with alternate injections of cells from two human multiple myeloma (MM) cell lines. Screening by the enzyme-linked immunosorbent assay (ELISA) technique showed that MM4 reacted with human MM cell lines (7 of 7 positive), as well as bone marrow aspirates from MM patients (4 of 4 cases positive). MM4 did not react with marrow aspirates from control patients (3 cases), or with peripheral blood mononuclear (PBM) cells from normal subjects, lymphocytic (12 cases) and myelogenous (8 cases) leukemia patients. In addition, MM4 was negative with polymorphonuclear leukocytes and RBCs from normal donors. By means of the immunoperoxidase technique, the MM4-reactive antigen was detected in paraffin-embedded, Zenker formalin-fixed bone marrow biopsies of MM (12 of 12 cases positive),

Waldenström's macroglobulinemia (2 of 2 cases positive), asymptomatic plasma cell dyscrasia (4 of 4 cases positive), and certain lymphomas (2 of 5 cases positive). Marrow biopsies from lymphocytic (5 cases) and myelogenous (5 cases) leukemias were uniformly negative. The MM4-reactive antigen also was expressed on plasma cells generated from pokeweed mitogen (PWM)-stimulated normal PBM cultures. The pattern of reactivity of MM4 with lymphocytes of B origin was similar to that of the plasma cell MoAb PCA-1. Competitive binding studies showed, however, that these two MoAbs recognized distinct antigenic determinants. These observations suggest that MM4 may be useful for the study of human plasma cell dyscrasias.

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MULTIPLE MYELOMA (MM) is a malignant lymphoproliferative disorder of B cells at their terminal stage of differentiation. The etiology of this disorder is poorly understood.¹⁻⁴ The wide spectrum of clinical manifestations of MM is the result of uncontrolled clonal proliferation of the myeloma cell.¹⁻⁷ However, antigenic specificity of the malignant clone is generally difficult to establish.⁴⁻⁶ It is also unclear why certain patients with plasma cell dyscrasias (PCDs) progress to malignant myeloma, whereas others remain in a stable nonprogressive state (benign monoclonal gammopathy).⁶⁻⁸ Since alkylating agents became available for therapy 20 years ago, the prognosis for overt MM has not improved substantially.^{1,2,5-8}

Through hybridoma technology, phenotypic studies of tumor cell types have contributed to a better understanding of certain hematologic malignancies.⁹ Characterization of tumor- or differentiation-antigen expression in leukemias and lymphomas has proven valuable for tumor diagnosis, classification, and prognosis.^{9,10} This approach also has provided the basis for tumor cell-specific targeting in immunotherapy trials.¹¹ By comparison, the immunologic phenotype of the myeloma cell is incompletely understood. The

myeloma cell and its normal counterpart, the plasma cell, lack such common B cell antigens as surface immunoglobulins, Ia, or complement receptors.^{1,10,12} B cell differentiation antigens such as B1, B2, B4, and B5 are also absent.¹⁰⁻¹⁴ Pronounced heterogeneity at the cellular and ultrastructural levels are common in MM.^{2,5}

In an attempt to characterize antigens on the myeloma cell better, we produced a monoclonal antibody (MoAb), MM4, that recognizes a commonly expressed antigen on human MM cell lines. MM4 also reacts with myeloma cells in bone marrow of patients with various forms of PCD, but not with peripheral blood mononuclear (PBM) cells, polymorphonuclear neutrophils (PMN), RBCs, or bone marrow (BM) cells from normal subjects and control patients. The selective reactivity of MM4 makes it potentially useful for the further study of human PCD.

MATERIALS AND METHODS

Cell lines and monoclonal antibodies. The plasma cell lines GM 1312, GM 1500, and GM 1311 were obtained from the NIGMS Human Genetic Mutant Cell Repository, Institute of Medical Research, Camden, NJ. RPMI 8226, ARH-77, HS-Sultan, and the colon carcinoma cell line WiDr were obtained from the American Type Culture Collection, Rockville, MD. LICR/LON was kindly provided by Dr Denis Burger, VA Medical Center, Portland, OR.

The plasma cell-reactive antibody PCA-1 and peripheral blood B cell antibody B1 were obtained from Coulter Diagnostics, Hialeah, FL. OKT3, which identifies peripheral T lymphocytes, and the precursor/activated T cell MoAb OKT10 were obtained from Ortho Diagnostics Systems, Raritan, NJ. MoAbs to the pre-B cell antigen common acute lymphoblastic leukemia antigen (CALLA) and the Ia (HLA-DR-related Ia-like) antigen were purchased from Hybritech, La Jolla, CA. Anti-human immunoglobulin κ and λ light chain antibodies were products of Bethesda Research Laboratory, MD. Antibodies to human immunoglobulin (anti-Ig; heavy and light chain specific) were obtained from Kallestad Laboratories, Austin, TX.

Patient blood and BM specimens. Human PBM cells and BM samples from patients with hematologic malignancies or normal

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controls were procured according to the protocol approved by the Institutional Review Board for Human Protection, Baylor University Medical Center (BUMC).

Patients with hematologic malignancies were diagnosed and classified independently by the clinical staff, Department of Oncology, Charles A. Sammons Cancer Center, BUMC. Sections of paraffin-embedded BM biopsies from these patients were obtained from the Department of Pathology, BUMC, after routine histopathological evaluation of the specimens. Immunologic phenotyping studies on leukemic patient PBM cells by immunofluorescence were performed independently by the Diagnostic Immunology Laboratory, BUMC, as described previously.¹⁵

Production of MoAbs. MM4 was generated by an alternate immunization protocol.^{16,17} BALB/c mice were sensitized with four weekly injections of 1×10^7 live cells, alternating with cells from the RPMI 8226 and GM 1312 human MM cell lines. Two booster injections were then given three days apart, alternating with cells from the two cell lines. Somatic hybridization of the sensitized splenocytes with SP2/0-Ag14 myeloma cells (1:1, splenocyte/myeloma cells) was performed 3 days after the last injection, using 50% polyethylene glycol and 5% dimethyl sulfoxide according to the method of Oi and Herzenberg.^{16,18}

Fusion products were cultured in hypoxanthine:aminopterin:thymidine (HAT) and IMDM (Iscoe's modified Dulbecco's medium, GIBCO, Grand Island, NY):15% fetal calf serum (FCS, GIBCO) at 37 °C in 5% CO₂/95% air. One hundred microliters of the cell suspension were dispensed into each well of five 96-well microtiter plates containing syngeneic thymocytes as "filler cells" (10^6 to each well).¹⁶ Supernatant fluids from confluent cultures were screened by ELISA for antibody activity against MM cell lines (RPMI 8226, GM 1312) and PBM cells from normal donors. Hybridomas with desirable reactivity were transferred to 24-well plates, grown to confluence (3 to 5 days) and then cloned twice by limiting dilution. Selected hybrid clones were carried in vivo by injecting 2×10^7 cells intraperitoneally (IP) in Pristane (Aldrich Chemicals, Milwaukee)-primed BALB/c mice.¹⁸

Ascitic fluids were harvested 7 to 10 days after induction. MM4 was partially purified by precipitation with saturated ammonium sulfate solution and adjusted to a concentration of 10 mg/mL.¹⁸

Peripheral blood cell separation. Fractionation of peripheral blood cells was carried out as described previously.¹⁹ RBCs were obtained by dextran (6%; mol wt >250,000) sedimentation (37 °C for 30 minutes) of heparinized whole blood. The leukocyte-rich fraction was resuspended to original volume with RPMI 1640 (GIBCO) and separated into PBM and PMN fractions by centrifugation with Hypaque-Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ).

Enzyme-linked immunosorbent assay. An enzyme-linked immunosorbent assay (ELISA) technique with intact target cells was used for antibody screening, as described previously.¹⁶ Incubation with the primary antibody was carried out at 37 °C for 60 minutes, followed by a peroxidase-conjugated secondary antibody (37 °C for 60 minutes; peroxidase-conjugated goat anti-mouse IgG + IgM, 1:3,000; TAGO, Burlingame, CA) with washings in between. The MoAb reactivity was determined as a function of enzyme reaction to the substrate *o*-phenylenediamine (37 °C for 30 minutes; Sigma, St Louis), measured by absorbance at 492 nm (Titertek Multiskan MC, Flow Laboratories, McLean, VA). Reactions of >0.1 OD U above background were considered positive.

Antibody isotyping. Subclass-specific rabbit anti-mouse immunoglobulins (Mouse Immunoglobulins Subtype Identification Kit, Boehringer Mannheim Biochemicals, Indianapolis) were used in an ELISA assay.¹⁶

Immunoperoxidase staining protocol. Zenker formalin-fixed,

paraffin-embedded BM biopsy sections were deparaffinized overnight, rehydrated, and treated with Lugol's iodine and 5% sodium thiosulfate.²⁰ The sections were washed in distilled water, then treated with 1% H₂O₂ in absolute methanol and normal horse serum. Immunoperoxidase staining involved 60-minute incubations at room temperature with the primary antibody, then secondary antibody (biotinylated horse anti-mouse IgG), followed by the enzyme avidin biotin-conjugated horseradish peroxidase (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and substrate (0.02% 3-amino-9-ethylcarbazole:0.03% H₂O₂:5% *N,N*-dimethylformamide), as described previously.¹⁶ Sections were counterstained with 0.125% methylene blue. Reactivity in each case was determined by light microscopy, in comparison with a section treated with the negative control MoAb SCLC 1096 that was generated against human small cell lung carcinoma.²¹ According to the frequency of reactive cells, reactivity with MoAb was graded as: -, no difference from background as compared with negative control; +, 5% to 10% of nucleated cells positive; ++, 10% to 20% of nucleated cells positive; and +++, >20% reactive cells.

Colony growth assay. The stem cell colony growth assay for the human myeloma cell line RPMI 8226 was performed as described previously.¹⁶ One hundred microliters of MM4 were incubated with 1×10^5 cells for one hour at room temperature. MoAb-treated cells in duplicate were distributed onto a six-well culture plate (Falcon, Oxnard, CA) in 0.3% agar:IMDM:15% FCS and placed in 37 °C incubator. The number of colonies (with >50 cells) was enumerated under low magnification after 7 days. The percentage of growth inhibition was determined by comparing MM4-treated cultures with those treated with the same concentration of an irrelevant MoAb SCLC 1096.

Proteolysis of RPMI 8226 cells. RPMI 8226 cells were washed twice and resuspended in IMDM at 1×10^7 cells/mL. Thirty-five microliters of pronase (Boehringer Mannheim) at 10 mg/mL were incubated with 700 μ L of cell suspension at room temperature for 1 hour.²¹ The reaction was terminated by washing twice with IMDM:15% FCS at 4 °C.

Biochemical characterization of MM4-reactive antigen. Cells from the RPMI 8226 cell line (2×10^7) were incubated with 0.2 mCi ³⁵S-methionine (New England Nuclear, Boston) in methionine-free media (RPMI 1640 Select-Amine kit, GIBCO) and 5% FCS for four hours.²² The labeled cells were washed twice in RPMI 1640 at 4 °C, resuspended in 2 mL of 0.5% NP40 (Sigma), 0.01 mol/L of Tris-HCl, pH 7.6, containing 1 mmol/L of phenylmethylsulfonyl fluoride (Calbiochem, La Jolla, CA) and 0.02% sodium azide, and kept on ice for 60 minutes. The cell lysate was cleared by centrifugation (10,000 g for 15 minutes), then snap-frozen at -70 °C until use.

Extraction of the ³⁵S-labeled MM4-reactive antigenic determinant was performed using MM4 conjugated to CNBr-activated Sepharose 4B (Pharmacia).²² Cell lysates were passed through the MM4-Sepharose or IgG-Sepharose columns (~50,000 cpm) at a flow rate of 0.1 mL/min. The columns were washed with 10 $\times$ vol of detergent lysate buffer. The antigen-bound gels were removed from the column, suspended in sample buffer (0.001 mol/L of EDTA, 1% sodium dodecyl sulfate, 5% 2-mercaptoethanol) and boiled at 100 °C for four minutes. Ten-microliter samples were introduced onto a 4% to 30% gradient slab gel (PAA 4/30, Pharmacia) side by side with radiolabeled markers of known mol wt (Pharmacia). Electrophoresis was carried out in Tris/acetate buffer (0.04 mol/L of Tris, 0.02 mol/L of CH₃COONa, 0.002 mol/L of EDTA, 0.2% SDS, pH 7.4) for two hours at 125 V, constant current (gel electrophoresis apparatus GE-4, Pharmacia). The gel was developed using Enlightening (New England Nuclear), dried, and then analyzed by autoradiography at -70 °C. The mol wt of the reactive determinant was

calculated by linear regression analysis as a function of its migration distance (Rf) in relationship to radiolabeled known mol wt markers.

Mitogen-stimulated cell cultures. Hypaque-Ficoll-separated PBM cells were suspended in RPMI 1640/10% FCS at 1×10^6 /mL. One milliliter of the cell suspension was incubated with PWM (final dilution 1:50, vol/vol; GIBCO) in 17×100 -mm polystyrene culture tubes (American Scientific Products) for the indicated time period.^{10,12}

Immunoperoxidase reactivity of PWM-stimulated PBM cell cultures was determined using Saccomanno fixative (50% ethanol/2% polyethylene glycol)-treated cells deposited on slides (5×10^5 cells to each slide) by cytospin centrifugation (Shandon Southern, Sewickley, PA).²³ Alternatively, MoAb reactivity was evaluated using the ELISA assay as described above.

Competitive binding assay. A radioimmunoassay was developed to compare MM cell binding by MM4 and PCA-1 (Coulter Immunology, Hialeah, FL). Diethylaminoethanol (DEAE)-chromatography-purified MM4 was labeled with carrier-free ¹²⁵Iodine (New England Nuclear) by the lactoperoxidase method.²⁴ The radiolabeled MoAb was separated from free iodine by being passed over a Dowex column (Dupont Chemicals, Boston),²⁵ dialyzed repeatedly in phosphate-buffered saline (PBS) and adjusted to a final protein concentration of 1 mg/mL according to absorbance at 280 nm. ¹²⁵I-MM4 retained >90% of the reactivity of unlabeled MM4, as determined by ELISA.

Competitive binding with ¹²⁵I-MM4 by unlabeled MM4, PCA-1, or the negative control normal mouse IgG was determined using a microtiter plate containing 5×10^4 attached RPMI 8226 cells to each well (as described in ELISA). Fifty microliters of unlabeled MM4, PCA-1, or normal mouse IgG (Cooper Biomedical, Malvern, PA) at molar ratios from 100:1 to 0.1:1 (unlabeled antibody/¹²⁵I-MM4) were added in triplicate (37 °C for 60 minutes). Target cells then were incubated with ¹²⁵I-MM4 (50 µL per well at 37 °C for 60 minutes) after being washed three times with PBS. ¹²⁵I-MM4 bound to each well was determined in an auto-γ scintillation spectrometer (Packard, Laguna Hills, CA) after the plate was washed five times with PBS. Optimal binding of ¹²⁵I-MM4 (cpm_{max}) was determined with RPMI 8226 cells that were not treated with unlabeled antibody, and compared with binding with unlabeled antibody-pretreated target cells (cpm_{treated}). cpm_{background} was established by determining ¹²⁵I-MM4 binding of normal peripheral blood PBM. The percentage of inhibition of ¹²⁵I-MM4 binding was calculated by the formula:

$$\left(1 - \frac{\text{cpm}_{\text{treated}} - \text{cpm}_{\text{background}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{background}}}\right) \times 100\%$$

RESULTS

Production of MoAb MM4. BALB/c mice were sensitized with alternate injections of cells from two MM cell lines, RPMI 8226 and GM 1312. This alternate immunization approach previously has been shown to enhance the generation of tumor-reactive hybridomas in a different human tumor system.¹⁶

More than 90% of wells developed hybrid clones within seven days of fusion. Supernatant fluids from 52% of these wells demonstrated reactivity with at least one of the two immunizing MM cell lines. Initial screening by ELISA showed that 15 wells contained clones reactive with both RPMI 8226 and GM 1312 cells, but not with normal PBM cells. These hybridomas were tested with other MM cell lines

and normal PBM cells. Hybridomas containing the desired reactivity were cloned twice by limiting dilution, then carried in mice peritoneally to generate antibody-rich ascitic fluids.

MoAb screening. To ensure that the MoAb of interest reacted with an antigen commonly expressed in MM, hybridomas were tested with seven different cell lines derived from peripheral blood (RPMI 8226, GM 1312, LICR/LON, ARH-77, and GM 1500), BM (GM1311), or plasmacytoma (HS-Sultan) of MM patients. These cell lines expressed either IgG κ or λ M-components (Table 1), with two of the seven lines (ARH-77, GM 1500) expressing Epstein-Barr virus nuclear antigen (EBNA).^{26,27} The hybrid clone MM4 reacted with all seven MM-derived cell lines tested, as determined by ELISA (Table 1). By comparison, the previously described plasma cell MoAb PCA-1¹² was positive with four of six cell lines tested.

MM4 did not react with PBM cells, RBCs, or PMNs from normal control subjects. The pooled ascitic fluid containing MM4 retained its selective reactivity at a dilution of 1/10,000. Subsequent studies were performed with ascites-derived MM4 that was partially purified by precipitation with a 50% (vol/vol) saturated ammonium sulfate solution.

The MM4-reactive antigen was expressed in BM aspirates from MM patients (4 cases), but was not detected in those from control subjects (normal donor, 1 case; colon carcinoma with no marrow involvement, 1 case). In addition, MM4 was unreactive with chronic myelogenous leukemia (CML) marrow (1 case). The plasma cell MoAb PCA-1 was similarly unreactive with normal PBM (0 of 7 cases positive) and control BM (0 of 2 cases positive), but reacted with BM from CML (1 of 1 case positive) as well as MM patients (4 of 4 cases positive).

Reactivity of MM4 with PBMs from patients with leukemia. Reactivity of MM4 also was examined using PBM cells from patients with lymphocytic or myelogenous leukemia. Their immunological phenotypes were defined by MoAbs to OKT3, OKT10, sIg, B1, Ia, and CALLA. MM4 was uniformly unreactive with PBM cells of chronic or acute myelogenous leukemias (Table 2). The antibody was also negative with T acute lymphocytic leukemia, as well as B leukemias that correspond to B cells at various stages of maturation. The latter included null cell, pre-B, and B acute

Table 1. Monoclonal Antibody Reactivity With Human Plasma Cell Cell Lines

Cell Line	M Component	ELISA Reactivity (OD U)*	
		MM4	PCA-1
RPMI 8226	IgGλ	0.689	0.713
GM 1312	IgGκ	1.038	0.103
LICR/LON	IgGκ	0.854	NT
ARH-77	IgGκ	1.040	0.091
HS-Sultan	IgGκ	0.893	0.144
GM 1500	IgGκ	0.455	0.115
GM 1311	IgGκ	0.462	0.002
No. positive/no. tested		7/7	4/6

NT, not tested.

*Determined by enzyme-linked immunosorbent assay (ELISA); OD values of ≥ 0.1 over background were considered positive.

Table 2. Myeloma Antibody Reactivity with Peripheral Blood From Patients With Leukemia

Diagnosis*	No. Positive/No. of Patients Tested†						Antibody Reactivity‡	
	T3	T10	Ig	B1	Ia	CALLA	MM4	PCA-1
Acute leukemia								
Lymphoblastic								
Null	1/2	2/2	1/2	0/2	0/2	0/2	—	NT
preB	2/2	2/2	1/2	2/2	1/2	2/2	—	NT
B	1/2	1/2	1/2	2/2	1/2	1/2	—	—
T	1/1	1/1	1/1	1/1	0/1	0/1	—	NT
Myelogenous	6/6	3/6	6/6	6/6	0/6	0/6	—	+
Chronic leukemia								
Lymphocytic, B	5/5	3/5	5/5	5/5	4/5	2/5	—	—
Myelogenous	2/2	2/2	1/2	1/2	0/2	1/2	—	+

CALLA, common acute lymphoblastic leukemia antigen. NT, not tested.

*Diagnosed independently according to clinical and laboratory findings.

†Determined by the Diagnostic Immunology Laboratory, BUMC; positive response represents $\geq 15\%$ Ab-reactive cells as determined by indirect immunofluorescence.‡Determined by enzyme-linked immunosorbent assay; OD values of ≥ 0.1 over background were considered positive.

leukemias, and B cell chronic lymphocytic leukemia (Table 2). By comparison, PCA-1 was also negative with chronic and acute B cell leukemias, but did react with myelogenous leukemia cells.

Immunoperoxidase reactivity of MM4 with BMs from patients with plasma cell dyscrasias. To delineate further the reactivity of MM4 among PCD, we examined its immunoperoxidase reactivity with Zenker formalin-fixed, paraffin-embedded BM biopsies from patients with MM, Waldenström's macroglobulinemia, or asymptomatic PCD (Table 3). The criteria for the diagnosis of MM were as previously

described.^{6,7} MM4 reacted with 12 of 12 MM BMs, and was equally reactive with marrow biopsies from patients with macroglobulinemia (2 of 2 cases positive) or asymptomatic PCD (4 of 4 cases positive). Reactive plasmacytes showed staining in the plasma membrane and cytoplasm. Nuclear staining was not observed.

To establish the mean frequency of MM4-reactive plasma cells within each PCD patient group, the percentage of MM4-reactive cells was compared with the frequency of plasma cells for each marrow biopsy. Of the 12 cases of MM examined containing 20% to 95% of plasma cells

Table 3. Immunoperoxidase Reactivity of Bone Marrow Biopsies From Patients With Plasma Cell Dyscrasias

Diagnosis	Case No.	Cellularity (%)	PC (%)	Immunoperoxidase Reactivity*	
				MM4	PCA-1
Multiple myeloma	1	40	75	++	+++
	2	90	50	++	++
	3	50	30	++	+
	4	100	95	+++	+++
	5	30	55	++	+
	6	30	90	+++	+++
	7	60	80	+	+
	8	80	40	+	+++
	9	50	40	+++	+++
	10	50	80	+++	+++
	11	60	20	+++	NT
	12	25	40	+	+
Waldenström's macroglobulinemia	13	100	50†	++	++
	14	75	55†	+	+
Asymptomatic plasma cell dyscrasia	15	35	10	+	—
	16	20	15	++	++
	17	70	20	++	+
	18	75	20	+++	+++

PC, plasma cells; NT, not tested.

*Immunoperoxidase reactivity was determined by the biotin-avidin complex reaction; the reactivity with each antibody was graded in comparison with that of a negative control antibody. The criteria used were as follows: —, no observable difference from control treated section; +, 5% to 10% of reactive nucleated cells; ++, 10% to 20% of reactive nucleated cells; and +++, >20% reactive nucleated cells.

†Majority plasmacytoid lymphocytes.

(57.9% $\pm$ 25%), 69.4% of plasma cells reacted with MM4, whereas 68.0% reacted with PCA-1. Similarly, 76.8% of the plasma cells in the asymptomatic PCD patient group reacted with MM4 (4 cases, percentage of PC = 16.3 $\pm$ 4.8), and 55.2% reacted with PCA-1. The two macroglobulinemia patients (mean percentage of plasmacytoid lymphocytes, 52.5%) contained 33% MM4-reactive and 22.8% PCA-1-reactive cells.

Immunoperoxidase reactivity of MM4 with BM biopsies from patients with leukemia and lymphoma. As compared with PCD marrow, MM4 reacted poorly with biopsies from leukemia patients. We observed no reactivity with chronic lymphocytic (2 cases) or myelogenous (1 case) leukemias. MM4 also was unreactive with acute lymphoblastic (3 cases) or myelogenous (4 cases) leukemias, whereas PCA-1 reacted with 2 cases from the latter group.

Both MM4 and PCA-1 were tested with bone marrows from patients with nodular (2 cases) and diffuse (3 cases) lymphocytic lymphomas. MM4 reacted with 2 of 3 cases of diffuse lymphocytic lymphomas (poorly differentiated lymphoma, 1 case; undifferentiated non-Burkitt lymphoma, 1 case). The same cases were positive with PCA-1, which, in addition, reacted with 1 of 2 cases of nodular poorly differentiated lymphocytic lymphoma tested. These observations indicate that the MM4-reactive antigen was expressed preferentially in PCDs and certain lymphomas.

MM4 reactivity with PWM-stimulated PBMs. Previous studies have shown that PWM induces differentiation of B cells to plasmacytes *in vitro*.^{10,12,13} To define expression of the MM4-reactive antigen in normal plasma cells, we examined its reactivity with PWM-stimulated normal cell cultures. Two other plasma cell-reactive MoAbs, OKT10 and PCA-1, together with the B cell MoAb B1 and anti-Ig light chain reagents (anti- $\kappa + \lambda$) were used for phenotyping of the cell cultures examined.

PWM cultures at days 0 and 4 expressed B1 (20% to 14%, respectively) and surface immunoglobulins (14% to 8%, respectively), as determined by the immunoperoxidase reaction with cytospin cell preparations (Table 4). These cells were minimally reactive with PCA-1, OKT10, or MM4. B1-positive cells, which included B cells from pre-B to mature presecretory stages of maturation, were significantly decreased at days 7 and 10. By contrast, these cultures from days 7 and 10 contained 10.8% to 22.5%, respectively, of MM4+ cells, and reacted also with PCA-1 (9.5% to 7.2%, respectively) and OKT10 (21.5% to 6.5%, respectively). Anti- $\kappa + \lambda$ reagents reacted with 19.4% to 25.5% of cells from day 7 and day 10 cultures. The reactive cells demonstrated pronounced cytoplasmic staining, as opposed to membrane-positive cells observed in day 0 and day 4 cultures. Morphologically, both MM4+ and PCA-1+ cells resembled plasma cells. Most contained eccentric nuclei and well-developed endoplasmic reticulum.

Competitive binding with ¹²⁵I-MM4. The pattern of reactivity of MM4 appeared to be similar to that of PCA-1, with the exception that MM4 did not react with normal or malignant granulocytes. A competitive binding assay was developed to determine whether these two MoAbs reacted with related antigenic determinants. ¹²⁵I-MM4 binding was

Table 4. Antibody Reactivity With Pokeweed Mitogen-Stimulated Normal Peripheral Blood Mononuclear Cells

Days in Culture*	Reactive Cells (%)†				
	B1	$\kappa + \lambda$ ‡	PCA-1	T10	MM4
0	20.9	14.3	1.7	0.4	0.9
4	14.2	8.2	0.7	0.3	1
7	5.9	19.4	9.5	21.5	10.8
10	2.9	25.5	7.2	6.5	22.5

*Peripheral blood mononuclear cells at 1×10^6 /mL with pokeweed mitogen at 1:50 (final dilution).

†Determined by immunoperoxidase reaction with cytospin cell preparations. Value represents number of positive cells in each 300 cells, duplicate determinations. Mean of two experiments is shown. Range of values was <25% of mean.

‡Comprised anti-human κ light chain antibody mixed with anti-human λ light chain antibody.

tested with RPMI 8226 cells that were preincubated with unlabeled PCA-1, MM4, or normal mouse IgG. As compared with untreated target cells, preincubation with unlabeled MM4 resulted in 25% to 82% inhibition of ¹²⁵I-MM4 binding, over a molar ratio (unlabeled: labeled MM4) range of 0.1:1 to 100:1 (Fig 1). These observations showed the capability of this assay in detecting antigen-specific competitive binding. Inhibition by MM4 was directly related to concentration of the preincubating antibody (regression analysis, $r = .97$, $F = 98.3$, $P = .000006$). By contrast, preincubation with PCA-1 or normal mouse IgG did not significantly affect ¹²⁵I-MM4 binding (PCA-1: $r = -0.29$, $F = 0.47$, $P = .52$) within the same molar ratio range of 0.1:1 to 50:1. The lack of competitive binding by PCA-1 suggests that MM4 and PCA-1 recognize distinct antigenic determinants.

Immunochemical characterization of MM4 and its antigen. MM4 was of the IgG1, κ isotype, as determined by the

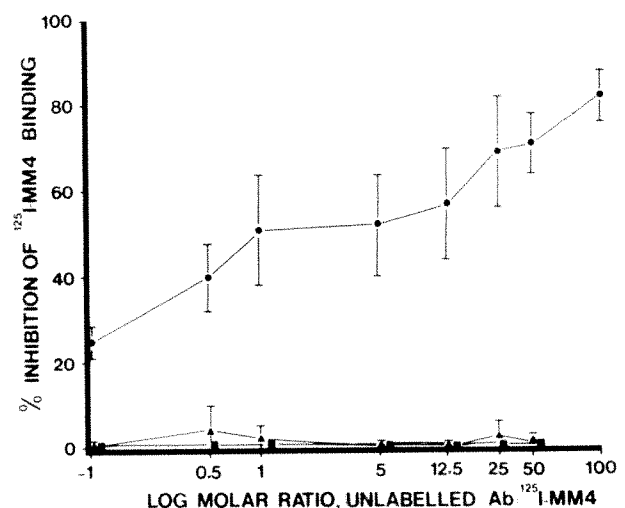


Fig 1. Competitive binding with ¹²⁵I-MM4. ¹²⁵I-MM4 binding with RPMI 8226 cells was determined by a radioimmunoassay after pretreatment of the target cells with MM4 (●), PCA-1 (▲), or normal mouse IgG (■) at the indicated molar ratio concentration (unlabeled antibody: ¹²⁵I-MM4).

ELISA assay using anti-mouse subclass-specific secondary antibodies.

The MM4-reactive antigen was protein in nature, as demonstrated by enzyme digestion studies. Treatment of the RPMI 8226 myeloma cells with pronase resulted in a loss of >95% MM4 reactivity as compared with untreated cells. Similarly, trypsin or chymotrypsin incubation resulted in 63% and 86% loss of reactivity, respectively. The reactive antigen on intrinsically ³⁵S-methionine-labeled RPMI 8226 was extracted by immunoprecipitation and analyzed using SDS-PAGE. Under reducing conditions, MM4 reacted with an antigenic determinant of ~39,000 to 40,000 mol wt (Fig 2).

Effect of MM4 on myeloma cell proliferation in vitro. To investigate the biological effect of MM4 binding, clonogenic stem cell growth of the MM cell line RPMI 8226 was examined after incubation with MM4.

Preincubation with MM4 resulted in a significant decrease in the number of myeloma cell colonies in day 7 cultures (Table 5). Exposure to MM4 at 1.0 mg/mL resulted in 75% to 90% reduction. This effect appeared to be dose-dependent, with a 50% reduction of colony formation after exposure to 0.01 to 0.001 mg/mL of MM4. MM4 did not inhibit colony formation of a nonreactive colon carcinoma cell line WiDr. Similarly, neither cell line was significantly affected by incubation with an irrelevant control MoAb small cell lung carcinoma 1096 (SCLC 1096) over the same antibody concentrations (data not shown), suggesting that inhibition of myeloma stem cell colony formation was directly related to specific binding of MM4.

DISCUSSION

This report describes the immunologic properties of a human myeloma cell-reactive MoAb, MM4, generated by an alternate immunization technique. This approach has been used previously in this laboratory to generate MoAbs to human SCLC tumor antigens.¹⁶ The alternate exposure of BALB/c mice to cells from two different SCLC cell lines promoted reactivity to antigens expressed in both immuniz-

Table 5. Inhibition of Colony Growth by Plasma Cell-Reactive Monoclonal Antibody MM4

Antibody Concentration (mg/mL)	Inhibition (%)*	
	RPMI 8226†	WiDr‡
1.0	81.4 ± 6.2	7.0
0.1	55 ± 18.5	0
0.01	48.9 ± 11.4	0
0.001	40.5 ± 20.9	1
0.0001	28 ± 17.0	0
0.00001	16 ± 9.0	4

*Cells 1×10^5 were treated with 100 μ L of MM4 antibody at room temperature prior to culture. Growth inhibition was determined at day 7.

†Myeloma cell line; value represents mean $\pm$ SD of three experiments.

‡Colon adenocarcinoma cell line; value represents mean of two experiments.

ing cell lines and apparently facilitated MoAb production against commonly expressed SCLC tumor antigens.¹⁶ We used the same rationale to produce MM-reactive MoAbs. To promote humoral immunity toward commonly expressed myeloma-associated antigens that were distinct from immunoglobulin or EBNA determinants, two MM-derived cell lines with different immunological properties were selected for immunization. RPMI 8226 was an IgG, λ light chain-secreting cell line,²⁸ whereas GM 1312 was derived from a IgG, κ myeloma.^{29,30} As is characteristic of myeloma-derived lines, both cell lines were morphologically pleomorphic, comprising cells with lymphoblastoid as well as plasmacytoid features.²⁶⁻³⁰ Most RPMI 8226 cells resembled immature plasma cells by electron microscopy,²⁸ and reacted with the plasma cell antibody PCA-1.¹² By comparison, GM 1312 cells in culture were less well differentiated morphologically, were unreactive with OKT10,³¹ and only weakly reactive with PCA-1. Approximately 38% of GM 1312 cells in culture expressed only cytoplasmic Ig, a feature consistent with plasmacytoid differentiation.³⁰ Unlike RPMI 8226, this cell line expressed EBNA determinants.^{28,30}

Based on reactivity with MM-derived cell lines, it was evident that the MM4-reactive antigen was not related to viral antigen or immunoglobulin light chain components. MM4 reacted with all seven cell lines tested, which comprised both IgG, κ and λ myeloma-derived cell lines that were either EBNA positive or negative. Furthermore, the MM4-antigen was not expressed on normal peripheral blood cells, as shown by negative MM4 reactions with PBM cells, RBCs, or PMN leukocytes.

Among malignancies of B lineage, null cell, pre-B and B acute leukemias, and B cell chronic lymphocytic leukemia corresponded phenotypically to transformed B lymphocytes at different stages of maturation.^{9,10} The demonstrated lack of reactivity with MM4 suggested that this antibody did not recognize the various common B antigens displayed by these cell types, such as receptors to C3, HLA class I or II framework antigens, surface Ig, or previously described B differentiation antigens, including CALLA, B1, B2, B4, and B5.⁹

Immunoperoxidase determinations with BM biopsies confirmed the cell line studies showing that the MM4-reactive

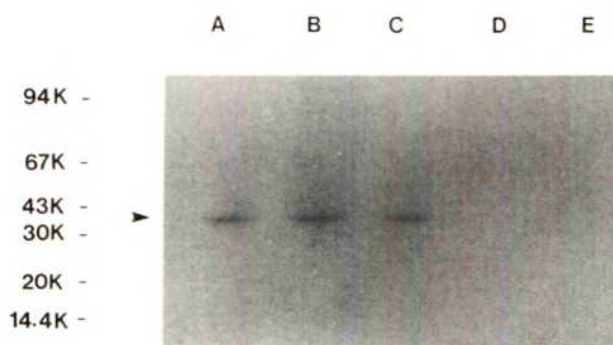


Fig 2. Analysis of MM4-reactive determinants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Reactive antigens derived from ³⁵S-methionine-labeled cell lysates were extracted by immunoprecipitation with MM4-Sepharose 4B. Lanes A through C, immunoprecipitate with MM4; lanes D and E, immunoprecipitate with normal mouse IgG. Arrow indicates MM4-reactive determinant of 39 to 40 kd.

antigen was commonly expressed on the plasma membrane and in the cytoplasm of myeloma cells. MM4 reacted with BM from MM and other PCDs, including Waldenström's macroglobulinemia and asymptomatic PCD. A lack of MM4 reactivity with PBM cells may reflect an absence of peripheral blood involvement.^{2,3,5,6,32}

Of the limited number of lymphoma cases examined, MM4 reacted only with diffuse lymphocytic lymphomas (two of three cases positive) of undifferentiated non-Burkitt, and poorly differentiated lymphocytic histology. The undifferentiated non-Burkitt tumor represents a pleomorphic small cell lymphoma that is phenotypically similar to transformed lymphoblastoid B cells.³³ Diffuse poorly differentiated lymphocytic lymphomas are related to midstage B cells, but commonly exhibit considerable heterogeneity with respect to cellular differentiation and phenotype.^{5,10} Both MM4-reactive lymphoma cases were also positive with PCA-1 and comprised pleomorphic cell populations with marked variations in cell size and nuclear contours. The MM4-reactive cells in each biopsy were plasmacytoid in appearance. Because of the small number of cases examined, it was unclear if these represented a terminally differentiated subset within the lymphoma cell population or infiltration with normal plasma cells.

Like the differentiation antigens found on early or mature B cells,⁹⁻¹⁴ the few plasma cell antigens described thus far have been detected on both normal and malignant cell types. The MoAb PC-1 described by Anderson and co-workers¹³ reacted with a 28-kd protein in normal and malignant plasma cells. Similarly, several plasma cell-reactive MoAbs described by Gonchoroff and co-workers³⁴ recognized antigens expressed on PCD marrow but not on B-CLL or normal PBM cells. Other MoAb-defined plasma cell antigens include the OKT10-reactive 45-kd glycoprotein found also on normal and activated T cells,³⁵ and the PCA-1 and PCA-2 antigens, which were expressed also on activated T cells, granulocytes, and monocytes, but not on normal B or T cells.¹² Similarly, the MM4-reactive antigen was expressed on plasmacytoid lymphocytes derived from PWM-stimulated cultures. Cells from these short-term cultures reacted poorly with B1 at days 7 and 10 but were positive with MM4 as well as with PCA-1 and OKT10. Thus, the MM4-reactive antigen apparently was expressed on terminally differentiated normal plasma cells as well as their malignant counterparts, although it was absent on unstimulated peripheral blood B lymphocytes. Preliminary studies in our laboratory have shown that MM4 reacts with cells from phytohemagglutinin and concanavalin A-stimulated PBM cultures. Unlike antigens that are PCA-1 or OKT10 reactive, however, the MM4-reactive antigen was not expressed on normal

T cells. The prevalence of plasma cell-associated antigens (as defined by OKT10, PCA-1, and PCA-2) on activated T cells is intriguing. Further experiments will be performed to establish the phenotype of the MM4-reactive cells in T cell mitogen cultures.

Pleomorphism among the monoclonal myeloma cell population is a well-documented phenomenon.^{2,3,5} At the cellular level, heterogeneity can be defined by antigen expression,^{2,10} cell ploidy,^{2,5,6} cell growth kinetics^{3,33} and susceptibility to chemotherapy.^{1,5,8} Although the MM4-reactive antigen was clearly evident in all MM marrows examined, only a portion (69.4%) of the total myeloma cells in each case expressed this antigen. The frequency of MM4-positive cells appeared to be similar among patients with MM and asymptomatic PCD. In contrast, marrow samples from macroglobulinemia patients contained a lower frequency of MM4-reactive cells (33%). This may reflect the smaller number of typical plasma cells in this patient population. The frequency of MM4-positive myeloma cells was similar to that of PCA-1-reactive cells in each patient group. Presently, it is not clear whether these two MoAbs recognize the same subset of myeloma cells. Although the two MoAbs demonstrated a similar pattern of reactivity, competitive binding experiments suggest that they recognize distinct antigenic determinants.

Expression of the MM4-reactive antigen on normal plasma cells, and in apparently equal proportions of MM and asymptomatic PCD cell populations, limits its usefulness as a marker for the diagnosis and classification of PCD. In vitro studies indicated, however, that MM4 binding can inhibit myeloma colony growth in vitro. Karp and colleagues showed that clonogenic colony-forming cells from MM marrow are closely related to kinetically active myeloma cells that proliferate in vivo.³⁶ A plasma cell MoAb such as MM4 that reacts with the stem cell subset thus may be potentially useful in the regulation of myeloma cell growth in vivo or for purging myeloma cells in autologous BM transplantation therapy of MM.³⁷ The efficacy of MM4 in eradicating myeloma cells from BM is currently under investigation in this laboratory.³⁸

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In Vitro Regulation of Human Hematopoiesis by Natural Killer Cells: Analysis at a Clonal Level

By F. Herrmann, R.E. Schmidt, J. Ritz, and J.D. Griffin

We studied the effects of a series of well-characterized clones of human natural killer (NK) cells on the proliferation of highly purified normal marrow hematopoietic progenitor cells. Individual NK clones suppressed granulocyte, monocyte, erythroid, or mixed colony formation in a heterogeneous but clonally stable manner. Inhibition of colony growth required a period of close cell contact between NK cell and progenitor cell with maximum inhibition occurring after 8 to 18 hours of preincubation time. The mechanism of killing was at least partially humoral, however, as

cell-free supernatants generated by NK clones "activated" by contact with a target cell also inhibited progenitor cell growth. One of the possible humoral mediators was identified as γ -interferon by studies with specific neutralizing monoclonal antibodies. These results show that clonal NK lines can be further activated by coming in contact with hematopoietic progenitor cells, resulting in substantial inhibition of colony formation in vitro.

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GROWING EVIDENCE shows that hematopoiesis both in vitro and in vivo can be modulated by certain "accessory" cells, including T cells, monocytes, and marrow stromal cells. Humoral factors released by these cells that either inhibit or promote proliferation of selected subpopulations of hematopoietic progenitor cells have been identified (reviewed in refs 1 and 2). Relatively little is known about direct cell-cell interactions between accessory cells and progenitor cells. In this regard, the possibility that natural killer (NK) cells may be involved in the regulation of hematopoiesis has been suggested both by in vivo studies of murine bone marrow transplantation and in vitro murine and human colony assays.³⁻⁷ The evidence that NK cells may participate in the regulation of human hematopoiesis is indirect. Leukemic proliferations of cells with NK-like activity have been reported in which anemia or neutropenia is a major component of the illness. Several studies have indicated that colony-forming progenitor cells are sensitive to NK-mediated inhibition to a variable extent,⁷⁻¹¹ although in other studies no inhibition has been detected.^{12,13} There have been conflicting reports on the sensitivity of various hematopoietic progenitor cells to NK-cell-mediated lysis, however,⁸⁻¹¹ and stimulation of certain progenitor cells has been reported.^{12,13}

The reasons for the variability of results are uncertain, but

possible contributory factors include heterogeneity of the NK cell population itself,¹⁴ differences in NK cell purification, different states of NK cell activation, and the variable presence of endogenous accessory marrow cells. In an effort to resolve some of the controversies over the effects of NK cells on in vitro human hematopoiesis, we studied the effects of a series of well-characterized clones of human NK cells on the proliferation of highly purified marrow colony-forming cells. These NK clones were selected to represent the various NK cell phenotypes present in normal peripheral blood. The results show that NK clones do not promote hematopoietic colony growth and that individual NK clones suppress subpopulations of progenitor cells in a heterogeneous but clonally stable manner.

Previous studies on the mechanism of NK cell lysis have implicated both direct cell-cell mechanisms and release of a humoral cytotoxic factor, NKCF.¹⁵ We therefore investigated the mechanism of inhibition of CFU-GM by active NK clones. The generation of an inhibitory effect required cell-cell contact, and maximum inhibition was observed only after 8 to 18 hours preincubation time with the target population. The inhibitory activity of NK clones was at least partially mediated by a humoral factor, since similar effects were seen when cell-free supernatants generated by suppressive NK clones activated by contact with progenitor populations were tested. We present evidence that NK-derived colony inhibitory activity (NKCIA) can be separated in part from NKCF, and finally that NKCIA can be partially, but not completely, inhibited by neutralizing monoclonal antibodies to interferon- γ (IFN- γ).

MATERIALS AND METHODS

Source of Human Cells

Normal peripheral blood and normal bone marrow (NBM) were collected into sterile, preservative-free heparin. All normal donors were advised of attendant risks of the procedures and gave informed consent in accordance with Institutional Review Board-approved procedures. The mononuclear cell fraction was separated by Ficoll-Hypaque gradient sedimentation. The BM donors were always unrelated to those individuals from whom NK cell lines were derived (see below). Mycoplasma-free leukemic human cell lines KG1 (myeloblasts), HL60 (promyelocytes), K562 (proerythroblasts) and Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell line Laz 461 and Laz 509 were maintained in RPMI 1640 medium

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supplemented with 10% fetal calf serum (FCS), L-glutamine, penicillin-streptomycin, and sodium pyruvate.

Partial Purification of Hematopoietic Progenitor Cells

Hematopoietic progenitor cells were enriched ~100-fold using an immune rosette technique previously described in detail.¹⁶ Monoclonal antibodies used in the purification of blasts included anti-MY8 (pan-myeloid), anti-Mol (clone 904, pan-myeloid), anti-Mo2 (monocyte), anti-B1 and anti-B4 (pan B), anti-T11 and anti-T3 (T), anti-NKH1 (NK) and anti-NKH₂ (NK and basophils), anti-glycophorin A (clone 39, K. Sabbath, J.D. Griffin, unpublished observations, January 1985) and anti-J5 [common acute lymphoblastic leukemia (CALLA) antigen]. Progenitor populations obtained in this way were subjected to a second rosetting step involving selection of HLA-DR-positive cells with I2 monoclonal antibody¹⁷ to improve the enrichment of CFU-GM. In experiments assaying for CFU-E, BFU-E, and CFU-Mix, depletion of glycophorin A-expressing cells was omitted and a second rosetting step was included using monoclonal antibody SC9¹⁸ to enrich for multilineage and erythroid progenitor cells. The 90-kd SC9 antigen is expressed by normal CFU-Mix, BFU-E, CFU-E, and erythroid cells less mature than the orthochromatophilic normoblast. CFU-GM are weakly positive, whereas more mature granulocytic cells are negative.

Generation of Cloned Human Cell Lines

Methods for generation of human NK cloned cell lines have been described in detail.^{14,19,20} In brief, either peripheral blood mononuclear cells (PBMCs) or purified NKH1+ or NKH2+ cells were cloned at one cell per well on a feeder layer of autologous, irradiated (5,000 rad) EBV-transformed B cells. Selected colonies with NK activity were expanded by the addition of culture medium containing lymphocyte-conditioned medium (LCM) (10% to 15% final dilution) every three days. Culture medium was RPMI 1640 supplemented with penicillin-streptomycin, L-glutamine, sodium pyruvate, and 20% human AB serum. All NK cell lines used in these studies have been subcloned at least four times at 100 cells per well on a feeder layer of irradiated PBMCs plus irradiated EBV-transformed B cells. After subcloning procedures, both phenotype and cytotoxic function have remained stable. As control cell lines, two cloned human cytotoxic T cell lines recognizing irrelevant antigens in a major histocompatibility complex (MHC)-restricted manner were kindly supplied by Dr Ellis Reinherz of Dana-Farber Cancer Institute.

Phenotypic Analysis of Cell Surface Antigens and Cytotoxicity of Cloned NK Cells

Phenotypic analysis was performed by indirect immunofluorescence with fluorescein-conjugated goat anti-mouse Ig (Tago, Burlingame, CA) as previously described.^{21,22} Monoclonal antibodies used for phenotype analysis of NK cells included T3, T4, T8, T11, NKH1, and NKH2. Samples were analyzed at 10,000 cells per sample on an Epics C flow cytometer (Coulter Electronics, Hialeah, Fla) using a log amplifier. As negative controls to determine background fluorescence, irrelevant ascites derived from nonreactive hybridomas were used. Monoclonal antibodies were always used at saturating concentrations (1:100 to 1:500). Cytotoxicity of NK clones was assessed using a standard ⁵¹Cr-release assay.¹⁴

Antibodies to IFN- γ and IFN- α

Polyclonal antibody to IFN- α and control antiserum was obtained from the Research Resource Branch, National Institutes of Health,

Bethesda, MD (catalogue nos. G-026-502-568 and G-027-501-568).

A monoclonal antibody (3C11C8), which neutralized recombinant and natural IFN- γ was raised against purified recombinant IFN- γ (the interferon was obtained from Biogen, Cambridge, MA).²³ Ascites fluid from mice bearing 3C11C8 hybridoma cells contained 10⁵ neutralizing U/mL. In all experiments, a second monoclonal antibody (2B4) that binds to IFN- γ but has no neutralizing capacity was used as a control.

Colony Assays

CFU-GM assay. CFU-GM were assayed in Agar (Agar Noble, Difco Laboratories, Detroit) by a modification of the method of Pike and Robinson.²⁴ Underlayers (0.5 mL) were composed of 0.5% agar in Iscove's modified Dulbecco's minimum essential medium (MEM) (IMDM) supplemented with L-glutamine and penicillin-streptomycin and 20% FCS. As a source of colony-stimulating factor (CSF), 10% conditioned medium from the GCT cell line (GIBCO, Grand Island, NY) was added to the underlayer. The overlayer (0.5 mL) was composed of 0.3% agar in the same medium and contained 2.5×10^3 purified myeloid progenitor cells in the presence or absence of 2.5×10^4 cloned NK cells. The NK cells do not proliferate under these culture conditions. The cultures were set up in quadruplicate in 24-well plastic culture plates (Linbro) and were incubated at 37 °C in 5% CO₂ and humidified air. In selected experiments, various anti-IFN- α and IFN- γ (at 1,000 to 3,000 nU/mL) or control antibodies were added to underlayers and overlayers. After 7 and 14 days of culture, overlayers were removed from underlayers by agitation and were dried onto glass slides under filter paper. To determine colony (>40 cells) and cluster (8 to 40 cells) morphology, the dried cultures were fixed in acetone-methanol fixative (60% acetone, 10% methanol, 30 mmol/L of sodium citrate, pH 7.4) for 1 minute, and were then stained for combined naphthol AS-D chloroacetate esterase (CAE) and α -naphthyl acetate esterase (ANAE) to detect granulocyte, granulocyte/monocyte, and monocyte colonies as previously described.²⁵ All slides were counterstained with acid hematoxylin (Sigma, St Louis).

CFU-Mix assay. Mixed granulocyte, erythroid, monocyte, and megakaryocyte colonies were assayed as described.²⁶ Progenitor cells were cultured in triplicate in 1-mL cultures in IMDM containing 0.9% methylcellulose, 30% FCS, 10% preselected conditioned medium from phytohemagglutinin (PHA)-stimulated (day 7 culture) PBMCs (PHA-LCM) free of detectable interferon (assayed by induction of class II HLA antigens on CFU-GM),²⁵ 2×10^{-5} mol/L of 2-mercaptoethanol and 1 U/mL of erythropoietin (Step III, Connaught Laboratories, Swiftwater, Ontario). Progenitor cells were plated at 1×10^3 to 1×10^4 /mL in 35-mm culture Petri dishes (Lux, Newbury Park, CA) in the presence or absence of 10⁵/mL of cloned NK cells. Cell concentrations were chosen so that there were 100 total colonies in each plate to minimize the possibility of colony overlap. In selected experiments antibodies to IFN- γ and IFN- α or control antibodies were added at the time of initiation of cultures which were scored on day 14.

CFU-E, BFU-E assay. Erythroid colonies and bursts were grown in triplicate as described²⁷ in 1-mL cultures of IMDM containing 0.9% methylcellulose, 30% FCS, 0.9% deionized bovine serum albumin (BSA), 2×10^{-4} mol/L of 2-mercaptoethanol, and 2 U/mL of erythropoietin. Erythroid progenitor cells were plated at 5×10^3 /mL in the presence or absence of 5×10^4 /mL cloned NK cells. Well-hemoglobinized colonies on day 7 of culture were scored as CFU-E; late hemoglobinizing bursts were scored as BFU-E at 14 days.

CSF-dependent myeloid progenitor proliferation assay. Proliferation of myeloid progenitors was assayed as described.²⁸ In brief,

1×10^4 /mL of purified myeloid progenitors were cultured in 0.2 mL of RPMI 1640 containing 10% FCS and various concentrations of NK-cell-derived supernatants prepared as described below for 48 hours in round-bottom microtiter 96-well plates (Linbro). In some cultures, GCT-conditioned medium (GIBCO) was added (10% vol/vol) as a source of CSF. Four hours prior to cell harvest, 20 μ L of RPMI 1640 containing 1 μ Ci of ^{3} H-thymidine (TdR) (Schwartz-Mann, Spring Valley, NY) was added. Cells were harvested on an automatic cell harvester (Microbiological Associates), and ^{3} H-TdR incorporation was measured in a Packard scintillation counter (Packard Instruments, Downer's Grove, IL). Assays were performed in triplicate, and results were expressed as mean $\pm$ SD.

Assay for proliferation of cell lines. Proliferation of cell lines KG1 and HL60 was assayed as described for myeloid progenitor cells, except that GCT medium was omitted from the cultures.

Preparation of cloned NK-cell-derived supernatants. Cloned NK cells washed free of their LCM propagation medium were plated either at 6×10^3 cells/well in IMDM containing 20% FCS, 1% GCT medium together with 6×10^2 purified NBM progenitor cells or KG1, HL60, K562, or Laz 509 cells in 96-well V-bottom plates (Linbro) in a total volume of 180 μ L/well, and centrifuged at 200 g for 10 minutes to initiate cell contact. For experiments in which contact was to be minimal, NK cells 2.4×10^4 /well were cultured along with 2.4×10^3 NBM progenitor cells in 24-well flat-bottom plates (Linbro) in a total volume of 0.72 mL/well. In both cases, cell concentration was the same (3.3×10^3 cells/mL). Plates were incubated at 37°C in 5% CO₂. Control cultures were performed under identical conditions in which NBM progenitor cells or KG1, HL60, K562, or Laz 509 cells were omitted. After 18 hours of incubation, cell-free supernatants were collected. In some experiments, aliquots of these supernatants were then cultured for 1 hour at room temperature in the presence or absence of 1,000 nU/mL of monoclonal antibody to IFN- γ (3C11C8) or control antibody.

Assay for colony inhibition by cloned NK cells. To test inhibition of hematopoietic progenitor growth, the different cloned NK populations were cultured at 2.5×10^4 to 10^5 /mL with purified hematopoietic progenitor cells in IMDM-20% FCS, 1% GCT in a NK/progenitor ratio of 10:1 in either of two culture conditions. To maximize cell contact, cells were centrifuged to a pellet (200 g for 10 minutes) in V-bottom microtiter plates and then incubated at 37°C for 4 to 18 hours. To minimize cell contact, cells were cultured in 24-well flat-bottom plates (Linbro) in a final volume of 1 mL. After the desired culture period, cells were transferred together with their culture supernatant into colony assays.

To test NK cell promotion of hematopoietic progenitor growth, cells from different NK clones, washed free of PHA-LCM, were incorporated directly into the various colony assays; exogenous growth supporting factors were not added.

RESULTS

NBM Progenitor Cells

The partial purification of NBM myeloid progenitor populations using a sequential two-step immune rosette technique resulted in preparations of 57% to 73% blasts, 20% to 28% promyelocytes, 1% to 3% more mature myeloid cells, 6% to 9% immature erythroid cells, and <1% lymphocytes and monocytes as previously described.²⁵ Reanalysis of the purified progenitor cells by indirect immunofluorescence using anti-Mo2, anti-B1, anti-T11, and anti-NKH1 revealed <1% fluorescent cells, indicating nearly complete absence of monocytes, B lymphocytes, mature T lymphocytes, and NK cells.

A second rosette step involving the selection of SC9-positive cells resulted in preparations of 28% to 40% blasts, 8% to 12% promyelocytes, 2% to 4% more mature myeloid cells, 49% to 53% immature erythroid cells, and <1% lymphocytes and monocytes.

Characterization of NK Clones

Six NK clones were selected for analysis. The phenotypes and cytotoxic activities of these lines are shown in Table 1, which shows that they were representative of the different types of activated peripheral blood NK cells. Retesting of these lines has shown phenotypic and cytotoxic stability for at least 10 months. Clones CNK8, JT9, and JT10 are T cell-like NK clones with T3/Ti-like structures, whereas clones CNK6, JT3, and JT₁₈ are T3 negative. All clones were shown to be positive for the pan-NK cell antigen NKH1_A, but only CNK6, JT3, and CNK8 reacted with NKH2, which defines a subset of large granular lymphocytes (LGLs) in peripheral blood. Two cytotoxic T3⁺ T cell clones that lack NK activity were included in some experiments as controls.

Regulation of Hematopoietic Colony Growth by Cloned NK Cells

NK clones were tested for colony stimulatory activity by using them as "feeder cells" in colony assays in which standard growth factors were omitted. None of the clones produced humoral factors that promoted the growth of CFU-GM, BFU-E, CFU-E, or CFU-GEMM (data not shown).

Pilot experiments with the CNK8 and JT10 clones revealed that co-culture of these NK cells with BM progenitor cells for 12 hours diminished subsequent CFU-GM colony numbers (Table 2) and that this effect was abrogated by prior irradiation of the NK cells (1,200 rad). Moreover, culture of marrow cells with two cytotoxic T cell clones that lack NK activity did not affect CFU-GM growth (Table 2). A series of experiments were then conducted to determine the culture conditions that resulted in optimum "killing" of CFU-GM, BFU-E, and CFU-GEMM by NK clones. The time of preincubation of NK cells and progenitor cells varied from 4 to 8 hours, the cell ratio varied from 2:1 to 20:1, and the cell contact conditions were either "loose contact" (24-well flat-bottom plate) or "tight contact" (96-well V-bottom plate). As shown in Fig 1, for two representative experiments assaying the effect of clone CNK8 on day 14 CFU-GM, CFU-E, and CFU-GEMM growth, colony inhibition required close cell contact and increased with longer preincubation time. In experiments not shown, the optimal NK/progenitor cell ratio was 10:1.

A series of five NK clones and two non-NK clones were then tested for inhibition of hematopoietic progenitor cells using the "optimum" culture conditions defined above (18 hours of co-culture with close cell contact at a 10:1 ratio) (Table 3). Two cytotoxic but non-NK T cell clones did not inhibit progenitor cell growth under these conditions (data not shown). In contrast, five of five NK clones tested inhibited growth of day 7 CFU-GM by >25% (range 27% to

Table 1. Phenotype and Cytotoxic Activities of Cloned Human NK Cell Lines

NK Clones	Phenotype*						Cytotoxicity†				
	T3	T4	T8	T11	NKH1	NKH2	K562	Laz 461	Laz 509	HL-60	KG-1
CNK6	—	—	—	+	+	+	+++	+	—	++	++
JT3	—	—	—	+	+	+	+++	+	—	+	++
JT ₈ 18	—	—	—	+	+	—	+++	++	—	++	+++
CNK8	+	—	+	+	+	+	+++	++	—	+	++
JT9	+	—	+	+	+	—	+++	+++	—	+	++
JT10	+	—	+	+	+	—	+++	+++	—	+	++

*Cells were considered negative if <10% of the cells and positive if >80% of the cells were reactive with the given monoclonal antibody.

†The cytotoxic activity is expressed as follows: —, <5%; +, 5% to 30%; ++, 30% to 60%; +++, >60%.

67%), whereas all clones inhibited day 14 CFU-GM by >25% (range 25% to 90%) (Table 3). All clones inhibited CFU-E growth by >25% (range 13% to 55%), whereas only three clones inhibited BFU-E by >25%. CFU-Mix were inhibited by >25% by three clones.

Inhibition of different colony types varied among individual clones. For instance, clone JT9, a poor inhibitor of CFU-Mix, strongly suppressed day 14 CFU-GM and CFU-E. It is notable that individual degree of inhibition was not associated with membrane expression of the anti-T3 or anti-NKH2 defined antigens, since clones with differential inhibitory effects were found in each phenotypic group. Moreover, the types of colonies inhibited by individual NK clones were reproducible from experiment to experiment and with marrow from different donors.

Agar CFU-GM cultures were stained in situ for esterase activities to determine if either granulocyte or monocyte colonies were preferentially sensitive to NK-mediated lysis (Table 4). At day 14, no preferential lineage inhibition of CFU-GM colonies was noted. Overall, granulocytic colonies were suppressed by 22% to 90%, monocyte colonies by 16% to 88%, and mixed granulocyte/monocyte colonies 25% to 89%. At day 7, however, the extent of inhibition of monocyte and mixed colonies (2% to 34% and 4% to 35%, respectively) was significantly lower than the inhibition of granulocyte colo-

nies (21% to 79% inhibition) ($P = .01$ and $.02$, respectively, based on two-sided paired t test) (Table 4), suggesting increased sensitivity of late granulocyte-committed CFU-GM to NK inhibition.

Inhibition of Myeloid and Erythroid Colony Growth by Humoral Factors Released by NK Cells

To investigate the mechanism of NK cell-mediated hematopoietic colony inhibition, we investigated the possibility that a soluble factor might be involved. Cell-free supernatants were obtained by incubating one of two selected NK clones with either purified progenitor cells or various cell lines under two different cell contact conditions (tight v loose cell contact). As shown in Table 5, supernatants derived from co-culture of progenitor cells with a clone (CNK8) that broadly inhibited hematopoietic colony growth subsequently suppressed day 14 CFU-GM colony formation by $58\% \pm 3\%$ and CFU-E formation by $41\% \pm 4\%$. Supernatants derived from co-cultures of an NK-clone (CNK6) that previously demonstrated modest inhibitory function with progenitor cells were much less effective in suppression of day 14

Table 2. Inhibitory Effect of Cloned Human NK Cell Lines on CFU-GM Growth

Cloned Cell Line*	CFU-GM/ 10^4 Cells†	Control (%)‡
T cell clones		
T01 5RexB	708	100
T02 20°C3	660	93
NK cell clones		
CNK8	380	54
JT10	208	29
NK cell clones (irradiated)		
CNK8	784	110
JT10	798	113

*Cloned cells were cultured with partially purified target cells for 12 hours in a V-bottom microtiter plate followed by cell suspension and assay of CFU-GM. The non-NK T cell clones are major histocompatibility complex-restricted allogeneic cytotoxic T cell clones of irrelevant antigenic specificity that lacked any demonstrable NK activity on K562 cells. Irradiation was 1,200 rad.

†Mean of quadruplicate values. SE < $\pm 20\%$ in each case.

‡The media control contained 708 CFU-GM/ 10^4 cells after 7 days.

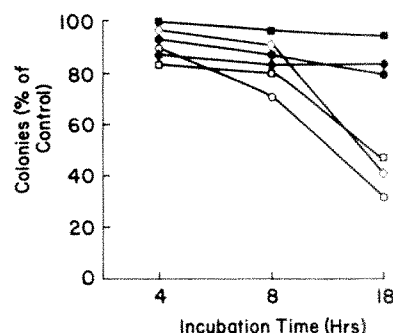


Fig 1. Influence of incubation time and degree of cell contact on suppression of day 14 CFU-GM, CFU-E, and CFU-Mix growth mediated by natural killer (NK) clone CNK8. Cells of NK clone CNK8 were cultured with partially purified hematopoietic progenitor cells (10:1) plated for various times either in 24-well flat-bottomed plates (CFU-GEMM, solid squares; CFU-E, solid diamonds; day 14 CFU-GM, solid circles) or in 96-well V-bottom plates (CFU-Mix, open squares; CFU-E, open diamonds; day 14 CFU-GM, open circles). Prior to incubation, 96-well plates were centrifuged to allow close cell contact. Flat-bottomed plates were not centrifuged. Mean $\pm$ SD of two separate experiments is shown. Absolute number of colonies/ 10^4 cells formed in triplicate cultures were: day 14 CFU-GM, 444 ± 72 ; CFU-E, 558 ± 56 ; CFU-Mix, 35 ± 2 .

Table 3. Inhibitory Effect of Cloned Human NK Cell Lines on Normal Bone Marrow Hematopoietic Colony Growth

Clone	Colonies (% of Control)*				
	CFU-GM† (Day 7)	CFU-GM (Day 14)	CFU-E	BFU-E	CFU-Mix
CNK6	73 ± 7	64 ± 5	64 ± 4	92 ± 5	79 ± 4
JT3	66 ± 7	75 ± 8	59 ± 4	50 ± 7	51 ± 6
JT ₈ 18	67 ± 5	38 ± 3	70 ± 9	70 ± 5	49 ± 6
CNK8	41 ± 5	20 ± 2	45 ± 6	51 ± 6	40 ± 3
JT9	33 ± 4	10 ± 3	49 ± 3	81 ± 6	80 ± 9

*The mean ± 1 SD of four separate experiments with four different normal bone marrow donors is shown.

†The absolute number of colonies (per 10⁴ cells) in triplicate cultures formed in the absence of cloned NK cells were: day 7 CFU-GM 788 ± 124; day 14 CFU-GM 436 ± 76; CFU-E 626 ± 140; BFU-E 150 ± 42; and CFU-Mix 34 ± 9.

CFU-GM (21% ± 4% inhibition) and CFU-E (13% ± 2% inhibition). Co-cultures of the same clones with sensitive and insensitive tumor lines revealed that supernatants produced by clones in the presence of sensitive tumor cell lines also contained CIA. Again, tight co-culture conditions resulted in more inhibitory effects (Table 5). CIA produced by CNK8 was more effective in colony inhibition following preincubation with hematopoietic progenitor cells than with sensitive tumor targets, suggesting that hematopoietic cells are particularly effective stimulators of this NK cell line.

Activation of NK Cells to Release Inhibiting Factors

The results shown in Table 5 suggested that "activation" of an NK clone by co-culture with a sensitive cell line induced release of inhibiting factors, whereas co-culture with an NK-resistant line was ineffective. The specificity of these inhibitory factors was further investigated by preparing supernatants conditioned by co-culture of CNK8 cells with either marrow progenitor cells, a resistant cell line (HL-60), or a sensitive cell line (KG-1), and then determining the effect of each supernatant on subsequent DNA synthesis (³H-TdR incorporation) of progenitor cells, HL-60, or KG-1 cells (Table 6). Marrow progenitor cells, composed primarily of granulocyte precursors, required the addition of CSF (GCT medium) for DNA synthesis.

Medium conditioned by unstimulated CNK8 cells had no inhibitory activity for progenitor cells, KG-1, or HL60 cells. In contrast, following stimulation with partially purified

marrow progenitor cells (MPCs), supernatants inhibited DNA synthesis of MPCs, KG-1 cells (NK sensitive) and HL60 cells (NK resistant). Following stimulation by KG-1 cells, supernatants inhibited MPCs and KG-1 cells, whereas stimulation by HL60 cells did not generate inhibitory activity. Supernatants generated following incubation with either MPCs or KG-1 cells were subsequently most effective at inhibiting the original target cell.

These results suggest that more than one inhibitory factor is released by activated NK cells. Because IFN-γ is known to be an effective inhibitor of hematopoiesis,^{25,29} experiments were performed in which the presence of IFN-γ in these supernatants was assessed by adding a neutralizing monoclonal antibody to IFN-γ (Table 6). The inhibitory activity of supernatants produced by CNK8 in the presence of myeloid progenitor cells, but not KG-1 cells, was partially neutralized by antibody to IFN-γ. Nonneutralizing antibody to IFN-γ, however, did not alter proliferation of the target cell populations tested (data not shown).

Role of IFN-γ Released by Cloned NK Cells in Inhibition of Hematopoietic Colony Growth

To analyze further the possible participation of IFN-γ apparently released by cloned NK cells on contact with hematopoietic progenitor cells in the inhibition of growth of various colony types, neutralizing antibody to IFN-γ was added to CFU-GM, CFU-E, BFU-E, and CFU-Mix assays at time of initiation of colony cultures.

The addition of 1,000 neutralizing U/mL of anti-IFN-γ partially abrogated the inhibition of day 7 granulocyte colonies mediated by CNK8 and JT9, but had minimal or no effects on other day 7 CFU-GM, day 14 CFU-GM, CFU-E, BFU-E, or CFU-Mix (Fig 2). In other experiments, the concentration of anti-interferon antibody was increased to 3,000 nU/mL without further reversal of the inhibitory effects of CNK8. These results suggest that IFN-γ is released as a consequence of the interaction between some NK cell lines and hematopoietic progenitor cells and that IFN-γ contributes to the cytotoxic effect of these NK cells on late granulocyte progenitor cells in particular. The failure of high concentrations of neutralizing monoclonal antibody to IFN-γ to abrogate completely the inhibitory effects of NK clones suggests, however, that additional factors are involved in the inhibition of hematopoiesis by these clones.

Table 4. Inhibitory Effect of Cloned Human NK Cell Lines on Subsets of CFU-GM

Clone	Colonies (% of Control)*					
	Day 7 CFU-GM			Day 14 CFU-GM		
	Granulocyte†	Monocyte	Mixed G/M	Granulocyte	Monocyte	Mixed G/M
CNK6	69 ± 5	84 ± 6	75 ± 7	65 ± 6	71 ± 5	68 ± 7
JT3	64 ± 5	66 ± 5	65 ± 6	72 ± 6	75 ± 8	75 ± 6
JT ₈ 18	59 ± 7	92 ± 7	80 ± 6	32 ± 3	45 ± 6	43 ± 2
CNK8	30 ± 4	80 ± 7	64 ± 5	24 ± 4	35 ± 3	31 ± 3
JT9	21 ± 3	70 ± 5	68 ± 4	10 ± 1	12 ± 3	11 ± 3

*The mean ± SD of three separate experiments with three different normal bone marrow donors is shown.

†The absolute number of colonies (per 10⁴ cells) in triplicate cultures formed in the absence of cloned NK cells were: day 7—CFU-gran 612 ± 64, CFU-mono 76 ± 16, and CFU-G/M, 100 ± 16; and day 14—CFU-gran 240 ± 36, CFU-mono 88 ± 24, and CFU-G/M 108 ± 24. Lineages of CFU-GM were determined cytochemically as described in the Materials and Methods section.

Table 5. Inhibitory Effect of Supernatants From Cultures of Cloned NK Lines CNK6 and CNK8 on Myeloid (Day 14 CFU-GM) and Erythroid (CFU-E) Colony Growth

NK Clone	Day 14 CFU-GM (% Colony Inhibition)*					
	Activating Cell Type†					
	None	KG-1	HL-60	K562	Laz 509	Purified Marrow Progenitor Cells
CNK6	2 ± 1	ND	ND	17 ± 4	3 ± 2	21 ± 4
CNK8						
Tight‡	2 ± 1	43 ± 4	7 ± 3	ND	ND	58 ± 3
Loose	1 ± 1	ND	ND	ND	ND	25 ± 3
	CFU-E (% Colony Inhibition)					
	Activating Cell Type					
	None					Purified Marrow Progenitor cells
CNK6		1 ± 1				13 ± 2
CNK8						
Tight		2 ± 2				41 ± 4
Loose		2 ± 2				19 ± 4

ND, not determined.

*The mean ± SD of two separate experiments is shown.

†NK cells (3.3×10^4 /mL) were cultured for 6 hours with 3.3×10^3 /mL of activating cells. Supernatants obtained from cultures of activating cells in the absence of cloned NK cells did not affect day 14 CFU-GM and CFU-E growth.

‡Supernatants obtained from tight co-cultures (*) and loose co-cultures (†).

DISCUSSION

We have investigated the role of NK cells and NK-derived soluble factors in regulating hematopoiesis in vitro by assaying the effects of cloned, activated human NK cell lines on the growth and differentiation of NBM colony-forming cells. Previous studies with these NK cell lines showed that they are useful models of activated human NK cells. They display considerable heterogeneity in the types of tumor target cells they recognize,²⁰ and some lines utilize the T cell antigen receptor (T3/ti complex) for target structure recognition, whereas other NK lines do not.³⁰ The results presented here

show that such NK cell lines do not promote formation of any hematopoietic colony type by themselves and do not release detectable soluble growth factors for colony formation. All clonal lines tested could inhibit one or more colony types in a reproducible manner, however. For most lines, colony inhibition was moderate (25% to 75% inhibition) and required a period of close cell contact of 8 to 18 hours for maximum effect. Finally, during this period of cell contact, soluble factors that mediate similar inhibitory effects are released. One, but probably not the only one, of these factors appeared to be IFN- γ .

Previous work on the regulatory properties of NK cells in

Table 6. Inhibitory Effects of Supernatants From Activated NK Cells on Proliferation of Normal MPC, an NK-Sensitive Tumor Cell Line (KG-1) and an NK-Resistant Tumor Cell Line (HL-60)

Target cell*	Culture	³ H-TdR Incorporation (cpm ± SD) per 1×10^4 Target Cells	Inhibition (%)	Inhibition (%) of ³ H-TdR Incorporation by Supernatant Pretreated With 1,000 nU/mL of Monoclonal Antibody to IFN- γ †
KG-1 (CNK8 sensitive)	CNK8 Sup	5,732 ± 638	—	—
	CNK8/KG-1 Sup	1,795 ± 163	69	67
	CNK8/HL-60 Sup	5,693 ± 701	1	0
	CNK8/MPC Sup	3,938 ± 307	32	14
HL-60 (CNK8 resistant)	CNK8 Sup	19,721 ± 1,442	—	—
	CNK8/KG-1 Sup	16,134 ± 1,539	18	18
	CNK8/HL-60 Sup	19,833 ± 1,348	0	0
	CNK8/MPC Sup	10,473 ± 1,099	47	27
Marrow progenitor cells	CNK8 Sup	984 ± 86	—	—
	CNK8 Sup + GCT	11,320 ± 1,240	—	0
	CNK8/KG-1 Sup + GCT	9,380 ± 1,007	18	12
	CNK8/HL-60 Sup + GCT	10,850 ± 1,039	4	2
	CNK8/MPC + GCT	3,720 ± 420	68	27

*Cells (1×10^4) were cultured in .2 mL of RPMI 1640 medium containing 25% (vol/vol) NK supernatants or medium alone for 48 hours prior to a 4-hour pulse with ³H-TdR. In all cases, culture with supernatant from unstimulated CNK8 cells was indistinguishable from culture with medium alone.

†Percentage of change of ³H-TdR incorporation on treatment with nonneutralizing monoclonal antibody to IFN- γ was consistently ± 2%.

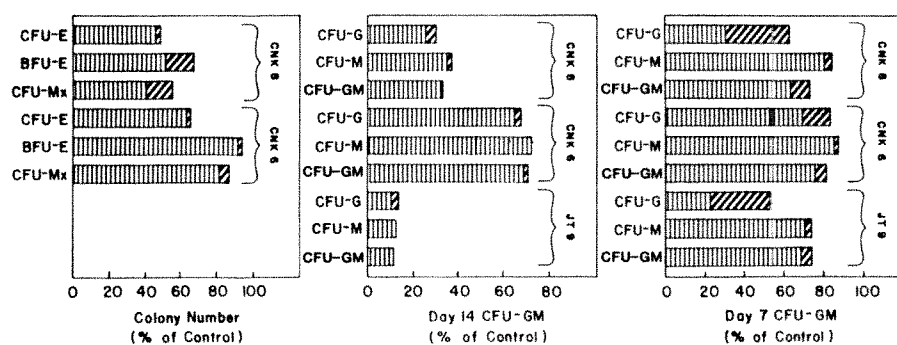


Fig 2. Influence of neutralizing monoclonal antibody to interferon- γ (IFN- γ) on inhibition of colony growth mediated by selected cloned natural killer (NK) lines. Panel A, day 7 CFU-E; panel B, day 14 CFU-GM; panel C, CFU-E, BFU-E, and CFU-Mix. Horizontal striped bar, control monoclonal (2B4); diagonal striped bar, 1,000 nU/mL of anti-IFN- γ (3C11C8). Results are presented as mean of triplicate culture from one experiment. A second experiment gave identical results.

human hematopoiesis has produced some conflicting results. By means of different experimental conditions, NK cells have been reported both to support and inhibit growth of the same hematopoietic colonies.⁸⁻¹³ Growth of peripheral blood-derived BFU-E has been reported to be promoted by soluble factors derived from unstimulated NK cells¹² as was growth of marrow CFU-GM in response to lectin-activated NK cells.¹³ Inhibition of marrow CFU-E was shown by Mangan and colleagues⁸ and Degliantoni and co-workers.⁹ Marrow-derived BFU-E were found to be suppressed by peripheral blood Leu 7 antigen-positive cells⁸ but not when NK cells were separated by selection of B73.1 antigen-positive cells.⁹ NK cells enriched by depletion of peripheral blood adherent cells and selection of the low-density fraction after Percoll density separation were strongly inhibitory to late (day 7) CFU-GM but were much less effective in inhibiting early (day 14) CFU-GM.¹³ In contrast, selection of B73.1-positive cells resulted in suppression of early CFU-GM without affecting late CFU-GM.⁹ These results already suggest that different enrichment procedures for NK cells may select for different NK subpopulations resulting in the observation of different functional activities. In addition, because hematopoietic colony-forming cells constitute only a small fraction of BM mononuclear cells (<0.5%), in many previous studies endogenous marrow accessory cells with potential inhibiting or stimulatory effects of their own (T cells, monocytes) far outnumbered the colony-forming cells. Thus, purification of the target populations (ie, marrow progenitor cells) may be a critical point in studying their direct interactions with NK cells *in vitro*. For example, it was recently demonstrated that NK cells may amplify endogenous T cells to become suppressor effector cells.³¹ In addition, residual monocytes could be stimulated by exogenous or endogenous CSF to release prostaglandins³² adversely affecting colony growth³³ and NK cell activity.³⁴ The effective removal of accessory cells and the higher concentration of progenitor cells in our experiments was designed to minimize these problems and allow determination of the direct effects of NK cells on progenitor cells.

We found that NK cell lines inhibited growth of all identifiable colony types, often with very modest degrees of inhibition (25% to 75%). Different clones tended to inhibit the formation of certain colony types preferentially, but not that of others. Although this was reproducible in these experiments, the differences among different colony types were often small, and we do not conclude from these data that activated NK cells specifically inhibit progenitor cells of

one lineage and not another, although that possibility deserves further investigation. Certain clones (CNK8 and JT9) displayed strong inhibitory capacity against most colony types, whereas others (CNK6) were minimally inhibitory in the same culture conditions. The lack of lineage-specificity of clones such as CNK8 suggests that the target "antigens" are not lineage restricted and that a possible candidate would be a proliferation-associated cell surface structure expressed by all hematopoietic progenitor cells. Although the role of NK-active cells in the regulation of hematopoiesis *in vivo* is unknown, these results suggest that certain NK cells can be activated by exposure to normal hematopoietic cells and can then inhibit proliferation of these cells. The results presented here suggest, however, that sensitivity to hematopoietic cells is not a property shared by all NK cells to an equal extent.

Other investigators have shown that supernatants from NK cells contain factors cytotoxic for tumor target cells (NKCFs) and may also contain inhibitors of colony growth.^{9,10} We investigated the role of inhibitory humoral factors in these studies with clonal NK cells by determining the degree of inhibition of various target cells by soluble factors generated by co-culture of NK cells with both sensitive and resistant tumor cell lines or with marrow progenitor cells. Our results confirm previous findings from Degliantoni and co-workers⁹ in that NKCFs released on contact with sensitive inducers exert inhibition of various colony types. More than one type of inhibitory factor was apparently generated, however, since NK cells activated by KG-1 cells secreted factors that were more effective in inhibiting KG-1 cell proliferation than marrow progenitor cell proliferation, whereas NK cells activated by progenitor cells secreted factors that were more effective in inhibiting progenitor cells than KG-1 cells. Purification of these factors would be of great interest but would be hampered by the limited amount of material obtainable from co-cultures with purified progenitor cell populations.

Because IFN- γ is known as a potent inhibitor of hematopoiesis^{25,26} a potent enhancer of NK cell function³⁵ and is known to be released from virus-induced NK cells,³⁶ we assayed for the presence of IFN- γ activity by neutralizing supernatants with specific anti-IFN- γ monoclonal antibody. Supernatants produced following culture of NK cells with hematopoietic progenitor cells but not with sensitive tumor targets contained inhibitory activity that could be partially neutralized by anti-IFN- γ antibody. It is presumed that the NK cells were the source of the IFN- γ rather than cells in the

progenitor cell fraction since IFN- γ was not detected in the absence of added NK cells, and the progenitor cell fraction should contain very few cells known to be capable of secreting IFN- γ (T cells, endogenous NK cells). If the IFN- γ is in fact secreted by the NK cell lines, these results suggest that different target cells may cause either different types of NK cell activation or, more likely, different degrees of NK cell activation. The different amounts of IFN- γ in NKClA and NKCFs could explain some of the diverse effects of these two supernatants. In colony assays, anti-IFN- γ but not anti-IFN- α or control antibody partially neutralized inhibition of late CFU-GM, and to a lesser extent BFU-E and CFU-GEMM, suggesting that other inhibitory mechanisms may be active in the inhibition of early CFU-GM and CFU-E. As shown, these may be of humoral nature, but since inhibitory effects due to cell-free

NKClA were less pronounced than those observed with NK cells, inhibitory activities related to direct cellular interactions may account for additional effects and have been the subject of further investigations (R.E. Schmidt, manuscript in preparation). The relevance of all in vitro NK assays in clinical situations is unknown. In the studies reported here, interpretation is further complicated by the fact that cloned NK cells represent highly activated cell populations that may have functional receptors distinct from resting NK cells. NK cells or activated NK-like cells may play a pathologically significant role in several clinical situations, however, including reconstitution following allogeneic BM transplantation,³⁷ the development of clonal hematopoietic disorders in NK-deficient Chediak-Higashi patients,³⁸ and in the development of anemia³⁹ and granulocytopenia⁴⁰ in patients with expanded NK cell, T- γ , or LGL populations.

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Flow Cytometric Analysis of Human Bone Marrow: I. Normal Erythroid Development

By Michael R. Loken, Virendra O. Shah, Karen L. Dattilio, and Curt I. Civin

Flow cytometry was used to identify maturational differences of erythroid lineage cells in normal human bone marrow by combining physical characteristics, the expression of multiple cell surface antigens, and nucleic acid content. Normal low-density bone marrow cells could be divided into four populations, based on forward and right-angle light scattering. Erythroid cells, at different maturational stages, were found in three of these four marrow subpopulations. The sequentially correlated expression of three cell surface markers—HLe-1, transferrin receptor, and glycophorin—allowed us to study erythroid maturation from the colony forming cell to the mature erythrocyte. HLe-1 was expressed on the earliest identifiable erythroid cells and was progressively lost as the cells matured. Transferrin receptor began to be expressed at the BFU-E stage and disappeared at the late reticulocyte stage. Transferrin receptor expression preceded glycophorin expression, the latter beginning on morphologically recognizable erythroid precursors just after the CFU-E stage. In

contrast to both HLe-1 and transferrin receptor, which were progressively lost during the maturational process, once glycophorin had been maximally expressed on the cell surface, it remained at constant quantities to the mature erythrocyte stage. Although developing nucleated erythroid cells at approximately the normoblast stage had light-scattering properties similar to those of lymphoid cells, these two cell types could be resolved by cell surface antigen expression. Normoblasts were glycophorin positive and HLe negative, whereas lymphoid cells expressed HLe and either Leu 4, Leu 11, or Leu 12. Decreases in cellular nucleic acid content, corresponding first to the extrusion of the nucleus and second to the loss of reticulum, characterized the later stages of erythroid development. These characteristics and instrumentation can be used to purify erythroid cells at various developmental stages.

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DIFFERENTIATION and maturation of hematopoietic cells is accompanied by changes in physical, biochemical, and antigenic characteristics that are specific for the various lineages. Previous studies of antigenic expression of bone marrow cells have used a single marker at a time, with little emphasis on the quantitative differences in antigen expression.¹⁻⁴ A more precise description of the continuous maturational sequence of cells can be obtained by correlating several parameters simultaneously in a quantitative manner (a technique applied to study lymphoid cells⁵⁻⁷).

In this study, we used three cell surface markers (glycophorin A, HLe-1, and transferrin receptor) in defining the stages of erythroid maturation in normal human marrow. Glycophorin A is expressed in large quantities on mature erythrocytes but is not present on the earliest precursor cells.^{8,9} In contrast, the HLe-1 antigen is lost during maturation.¹⁰ Transferrin receptor is expressed at intermediate stages of erythroid development.¹¹ Using flow cytometry to correlate the expression of these three cell surface markers with physical characteristics and nucleic acid content, we were able to identify erythroid cells at morphologically recognizable stages of maturation.

MATERIALS AND METHODS

Monoclonal antibodies. Phycoerythrin (PE)-conjugated Anti-Leu-4 (CD3), Anti-Leu-11 (CD16), Anti-Leu-12 (CD19), and rat anti-mouse κ , as well as Anti-HLe-1 FITC were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA. Anti-transferrin receptor (Becton Dickinson) was conjugated to PE following the protocol of Oi and colleagues.¹² Both purified and FITC-conjugated antiglycophorin (10F7) were the kind gifts of Dr R. Langlois, Livermore.¹³

Cell preparation and immunofluorescence. Bone marrow aspirates were obtained from normal volunteers. Collection of samples was performed after approval by the institutional Committee on Investigations Involving Human Subjects with an assurance filed with and approved by the Department of Health and Human Services. The low-density leukocytes ($1.077 \mu\text{g}/\text{cm}^3$, Ficoll-Hypaque, Pharmacia, Piscataway, NJ) were washed in RPMI 1640

containing 10% fetal bovine serum (FBS) and aliquoted for immunofluorescent staining. The cells were suspended at a concentration of $10^7/\text{mL}$. In the experiments in which both antibodies were directly conjugated to the fluorochrome, the first antibody (or mixture of antibodies, as in the experiments using PE Anti-Leu 4,11,12) was added using an amount that gave maximal fluorescence. The cells were incubated on ice for 30 minutes. They were then pelleted and washed with buffer (at least five times the staining volume) before the second antibody was added. After 30 minutes on ice the cells were again washed with buffer. For the experiment in which antiglycophorin was paired with anti-HLe-1, a four-step procedure was used. The cells were first labeled with antiglycophorin, as just described. Following the wash step, the cells were reacted with rat anti-mouse κ PE, incubated on ice, and washed. The cells were then exposed to 10% normal mouse serum for 30 minutes to block free binding sites of the second-step antibody. After the cells were washed with buffer, the anti-HLe-1 FITC was added and the cells were processed as before. After all the staining steps were completed the cells were resuspended to $2 \times 10^6/\text{mL}$ in RPMI 1640 containing 10% FBS.

Nucleic acid staining. Cells were labeled for cell surface antigens using directly FITC-conjugated antibodies and were fixed overnight in 1% paraformaldehyde in phosphate-buffered saline (PBS).¹⁴ The cells were pelleted and resuspended in saline containing 0.2% Tween 20 (Sigma, St Louis). After 15 minutes at 37 °C, the

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cells were washed once with PBS. An aliquot of cells was further exposed to 50 $\mu\text{g}/\text{mL}$ of ribonuclease A (Sigma) for 30 minutes at 37 °C. Both the untreated and RNase-treated cells were resuspended in 10 $\mu\text{g}/\text{mL}$ of propidium iodide (PI) for 30 minutes. The cells were washed with buffered saline once and were then analyzed immediately on the flow cytometer.

Flow cytometry. Quantitative fluorescence analysis was performed using a five-parameter FACS 440 (Becton Dickinson Immunocytometry Systems). The optical configuration and filter combinations for two-color immunofluorescence using FITC and PE have been previously described.¹⁵ In the experiments comparing FITC and PI staining, the optical filter in the PE channel was replaced by a 620 LP filter. The argon ion laser was operated at 488 nm using 100 mW of power. The laser beam was focused using a 2-inch focal length spherical lens. A minimum of 50,000 events was collected in list mode on a Consort 30 Data Management System. This permitted reanalysis of the data to demonstrate the correlation between two colors of immunofluorescence for population of cells identified by forward and right-angle light scattering. Contours were drawn at increments of five cells in the bivariate plots. The initial contour level for the populations identified as "Blast" by light scattering was 2. For all other contour plots, the initial level was 5.

The data presented in Figs 1 through 7 were all obtained from a single bone marrow sample in a single experiment. Replicate experiments gave similar staining results.

Morphological studies. Viable cells, stained for two colors of immunofluorescence, were sorted based on the gates indicated in the appropriate figures. Duplicate cytocentrifuge slides were prepared for each sorted fraction of cells. These slides were coded and stained with Wright's Solution, and differential counts made for each sample.

Enumeration of hematopoietic progenitors. The stained bone marrow cells were sorted directly into 24-well multiculture plates (Falcon Plastics, Oxnard, CA) containing 0.25 mL of growth medium consisting of 1% methylcellulose in Iscove's Modified Dulbecco's Medium (IMDM) (GIBCO, Grand Island, NY) with 30% FBS and 50 $\mu\text{mol}/\text{L}$ of 2-mercaptoethanol. For erythroid colonies, 1 U of erythropoietin and 1% Mo of conditioned medium¹⁶ were added, whereas for granulocyte-macrophage colonies 5% placental-conditioned media was added (kindly provided by Dr L. Levitt). The cultures were incubated at 37 °C in 5% CO₂. CFU-E colonies were counted on day 8; BFU-E colonies were counted on day 14. CFU-GM colonies were enumerated on day 11. Because the number of cells sorted into each well varied from 500 to 2,000 (depending on the relative enrichment of the progenitors expected), the number of colonies was expressed on the basis of 2,000 cells sorted for each well.

RESULTS

Physical parameters aid in discriminating between cell types. The forward and right-angle light-scattering contour plot of low-density Ficoll-Hypaque-separated normal human marrow (Fig 1) was similar to that obtained from blood in which the erythrocytes had been lysed using NH₄Cl.¹⁵ Windows L and G corresponded to the lymphocyte and granulocyte populations (respectively) observed in the analysis of blood. Blood monocytes were found in window B, which in the marrow was comprised of immature cells of various lineages, giving rise to the term "blast" window.¹⁷⁻¹⁹ Colony-forming cells were found almost exclusively within this region.¹⁷⁻¹⁹ Window R was unique to marrow and contained nonnucleated RBCs.

The cells within these windows were sorted and differen-

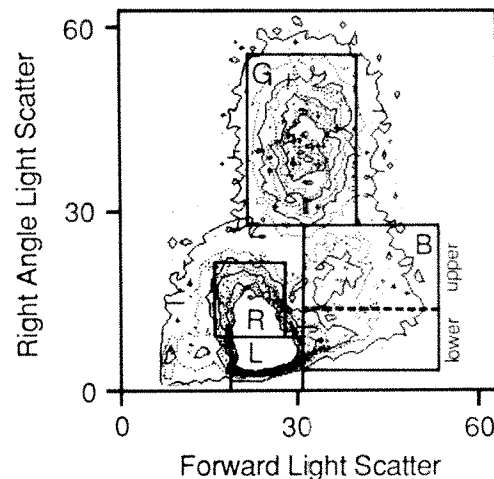


Fig 1. Correlation of forward- and right-angle light scattering of low density, viable human bone marrow. Four populations of cells (R, L, B, and G) were sorted for morphological identification (Table 1). These same gates were used for reanalysis of the list mode data to correlate the immunofluorescence with the light-scattering parameters of each cell.

tial counts of Wright's-stained cell preparations were made (Table 1). Mature, nonnucleated erythrocytes and reticulocytes predominated in population R. Less mature nucleated erythroid precursors (normoblasts) were enriched in population L, along with lymphoid cells. The least mature erythroid precursors identifiable by morphology were found in population B, which included immature cells of all other lineages. Window G contained almost exclusively maturing cells of the granulocytic lineage. Because Window G did not contain many erythroid cells, subsequent analysis in this study focused on the cell surface antigen expression of cells that were within windows R, L, and B.

Discrimination of erythroid and lymphoid cells. The mixture of erythroid and lymphoid cells found in windows R and L (Fig 1) could be distinguished based on cell surface antigen expression (Fig 2). Erythroid cells were specifically identified using FITC-conjugated antiglycophorin A.^{8,9} The lymphoid cells were identified as a group by mixing three PE-conjugated antibodies together; anti-Leu-4 (CD-3 on T cells), anti-Leu-12 (CD-19 on B cells), and anti-Leu-11 (CD-16 on NK cells).²⁰⁻²² As shown in Fig 2, 94% of all cells within the "RBC" light-scattering window were glycophorin⁺, whereas 6% were lymphoid, as defined by the expression of Leu 4, Leu 12, or Leu 11. In the "lymph" window, 75% of the cells were lymphoid, whereas 25% were erythroid. This was confirmed by FACS sorting and morphological analysis (data not shown). Because cells that were unreactive with either FITC or PE antibodies (box in Fig 2, lymph) constituted <0.5% of all cells within windows R and L (Fig 1), >99% of the cells that lie within these light-scattering windows were identifiable as either erythroid or lymphoid.

Common leukocyte antigen (HLe-1) is lost during erythroid development. The common leukocyte antigen (T200, identified by anti-HLe-1) was reported to be expressed on all leukocytes, including hematopoietic progenitor cells, but not mature RBCs.¹⁰ The relationship between

Table 1. Differential Morphological Cell Counts of Normal Human Bone Marrow Cells Sorted by Their Light-Scattering Properties

Windows*	RBC			Lymph			Blast			Gran		
Experiment†	I	II	III	I	II	III	I	II	III	I	II	III
Proportion in window‡	28	31	18	19	26	26	5	4	7	24	14	37
Cell type§												
Mature erythrocyte	77	63	39	1	6	6	2	4	1	0	0	0
Erythrocyte with polychromasia	23	32	58	0	0	0	0	0	0	0	0	0
Orthochromatic normoblast	0	0	<1	5	46	6	1	8	4	0	2	0
Polychromatophilic normoblast	0	0	<1	3	13	11	9	20	13	0	1	0
Basophilic normoblast	0	0	<1	0	8	0	11	9	10	0	0	0
Erythroblast	0	0	<1	0	2	1	27	12	26	0	0	0
Small lymphocyte	0	1	<1	56	20	24	0	0	4	0	0	0
Lymphoblast	0	0	<1	35	0	52	0	0	4	0	0	0
Plasma cell	0	0	0	0	0	0	5	0	2	0	0	0
Monocyte	0	0	0	0	0	0	0	4	5	3	0	0
Monoblast	0	0	<1	0	0	0	0	10	30	0	0	0
Mature neutrophil	0	0	0	0	0	0	0	0	0	0	1	23
Band	0	0	0	0	2	0	0	0	0	25	76	67
Metamyelocyte	0	0	0	0	1	0	0	0	0	46	13	9
Myelocyte	0	0	<1	0	0	0	0	5	0	10	0	1
Promyelocyte	0	0	<1	0	0	0	0	2	0	0	0	0
Myeloblast	0	0	<1	0	0	0	41	26	0	13	0	0
Eosinophil	0	0	0	0	1	0	0	0	0	3	0	0
Basophil	0	0	0	0	1	0	1	0	0	0	7	0

Lymph, lymphoid cells; Blast, blasts identified by light scattering; Gran, granulocyte.

*Cells were sorted based on their light-scattering properties as illustrated in Fig 1.

†Three replicate experiments using different bone marrow preparations were completed.

‡The proportion of cells that was classified in the windows expressed as a percentage of all cells.

§Cytocentrifuge slides were prepared from the sorted cell populations. These slides were stained with Wright's stain, and differential counts were made of the cell preparations.

the loss of HLe-1 and the acquisition of glycophorin A by erythroid cells during erythroid maturation offered a potential means to characterize the early stages of erythroid cell development. The bivariate analysis of normal bone marrow cells stained with antiglycophorin-PE and anti-HLe-1-FITC is shown in Fig 3. The correlation between the two antigens for all cells in normal bone marrow is shown in the "ungated" panel (Fig 3); for cells gated in the light-scattering windows identified in Fig 1 corresponding to windows R (RBC), L (lymph), and G (gran), it is shown in the other panels. Glycophorin⁺ erythroid cells were easily distinguished from the HLe-1⁺ lymphocytes in the RBC and lymph gates (Fig 3 as compared with Fig 2). As expected, no glycophorin⁺ cells were identifiable in the gran window.

The rather heterogeneous population identified as blasts by light scattering (Fig 1B) had a more complex relationship between glycophorin and HLe-1 (Fig 4). A population of strongly glycophorin⁺ cells was clearly identifiable (Glyco⁺⁺⁺, Fig 4). These Glyco⁺⁺⁺ cells expressed equivalent amounts of glycophorin as the mature erythrocytes found in the RBC light-scattering window. In addition, a small population of cells had intermediate amounts of glycophorin. These Glyco⁺ cells had higher quantities of cell surface HLe-1 antigen than did most of the Glyco⁺⁺⁺ cells (Fig 4).

The glycophorin⁻ cells could be subdivided into two populations which differed threefold in quantitative HLe-1 expression ("HLe-1⁺⁺⁺" v "HLe-1⁺⁺", Fig 4). The HLe-

1⁺⁺⁺ cells had more right-angle scatter signal than did the Glyco⁺⁺⁺ cells. Therefore, the data were reanalyzed with the blast window divided into upper and lower sections based on the right-angle scatter signals of the cells (dashed line in Fig 1B). The immunofluorescence of cells in the upper portion of window B is shown in the upper panel of Fig 4, whereas the staining of cells that were in the lower portion of window B are shown in the lower panel. The Glyco⁺⁺⁺ cells and the Glyco⁺ cells were predominantly in the lower portion of the blast window (Fig 1B). Very few cells that expressed glycophorin were found in the upper blast window. The remainder of the cells in the lower window were glycophorin⁻ and expressed intermediate quantities of HLe-1 (Glyco⁻, HLe-1⁺⁺, Fig 4). The glycophorin⁻ cells that were brightly labeled with HLe-1 (Glyco⁻, HLe-1⁺⁺⁺) were the predominant cell population in the upper portion in window B.

From these data, the cells exhibiting light-scattering parameters corresponding to window B, Fig 1, (which could not be delineated by the combination of antiglycophorin and antilymphoid markers) could be subclassified into four groups, based on the expression of glycophorin and HLe-1. Three populations were predominantly within the lower window of the blast gate (Glyco⁺⁺⁺, Glyco⁺, and Glyco⁻, HLe-1⁺⁺). The upper blast window contained cells of the fourth population (Glyco⁻, HLe-1⁺⁺⁺).

Transient expression of transferrin receptor. Transferrin receptor has also been reported to be expressed on

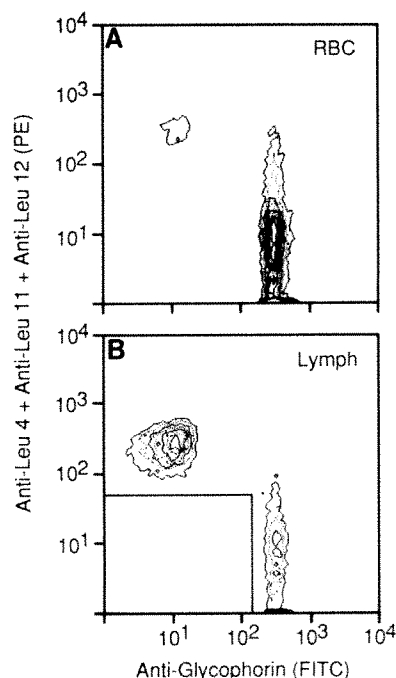


Fig 2. Discrimination between erythroid and lymphoid cells. The marrow cells were labeled with antiglycophorin FITC and a mixture of anti-Leu-4 (CD3), anti-Leu-11 (CD16), and anti-Leu-12 (CD19), all conjugated to phycoerythrin. The immunofluorescence of cells that had light scattering properties in windows R and L of Fig 1 are shown in (A) and (B), respectively. These two types of cells could be distinguished by cell surface antigens into two mutually exclusive groups. Less than 0.5% of the cells with light-scattering characteristics of either RBC or lymph were within the box denoted in (B), indicating that very few cells were negative for both of these fluorochromes.

early erythroid cells but is also expressed on other cell lineages.^{11,23-25} The correlations between the expression of transferrin receptor and HLe-1 on normal marrow cells are shown in Fig 5. A population of cells was identifiable which expressed neither the transferrin receptor nor HLe-1. As shown in Fig 5, all these transferrin receptor⁻, HLe-1⁻ cells had light-scattering properties in the RBC window and thus were erythrocytes. The majority of the cells within this light-scatter window, however, were transferrin receptor⁺, but were very dim for HLe-1 expression. A few HLe-1 brightly labeled (lymphoid) cells could be identified within this population. The cells that had light-scattering characteristics within the lymph window fell into two populations based on transferrin receptor and HLe-1 expression. The HLe-1 bright fraction (lymphoid, as shown in Fig 3) did not express transferrin receptor. In contrast, all the other cells in the lymph window expressed low amounts of HLe-1 (predominantly normoblasts, as shown above) and large quantities of the transferrin receptor.

The correlations between transferrin receptor and HLe-1 for cells within the blast window are also shown in Fig 5. The cell populations identified here are very similar to those identified by correlating antiglycophorin with anti-HLe-1 (Fig 4). The cells that expressed higher amounts of HLe-1 than did granulocytes also expressed transferrin receptor. By comparison with those in Fig 4, these latter cells were predominantly glycophorin⁻.

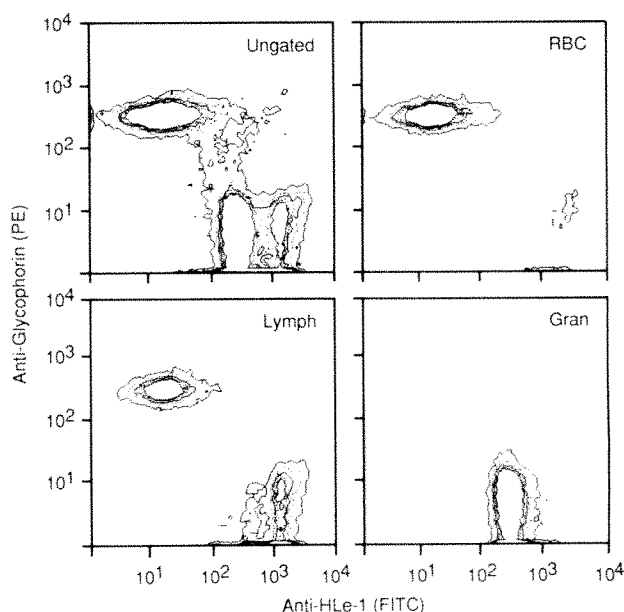


Fig 3. Correlation between glycophorin and HLe-1 expression on normal marrow. The multiple populations identified in the ungated panel could be identified by gating the data based on the light-scattering characteristics shown in Fig 1. The bright glycophorin⁺ cells were in the RBC and lymph (lymphocyte) windows but not in the gran (granulocyte) window. Note that the intensity of HLe-1 expression was different for cells in the lymph and the gran windows.¹⁰

Correlation of expression of transferrin receptor and glycophorin. The same bone marrow population was simultaneously studied with PE-conjugated antitransferrin receptor and FITC-conjugated antiglycophorin (Fig 6). The glycophorin⁺ cells within the RBC window (erythrocytes) were

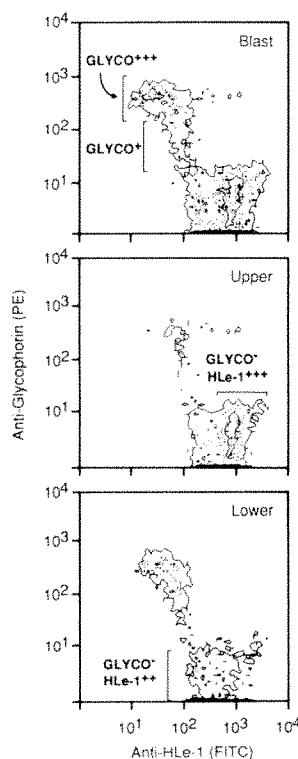


Fig 4. Correlation between glycophorin and HLe-1 expression of cells within window B, Fig 1. This "blast" light-scattering window was further divided into "upper" and "lower" portions (dashed line, Fig 1). Four populations were evident: (a) Bright glycophorin⁺ cells (Glyco⁺⁺⁺), predominantly in the lower window; (b) dim glycophorin⁺ cells (Glyco⁺), also in the lower fraction; (c) glycophorin⁻, HLe-1-intermediate labeled cells (HLe-1⁺⁺, Glyco⁻) (lower fraction); (d) glycophorin⁻, HLe-1 bright cells (Glyco⁻, HLe-1⁺⁺⁺) (upper fraction).

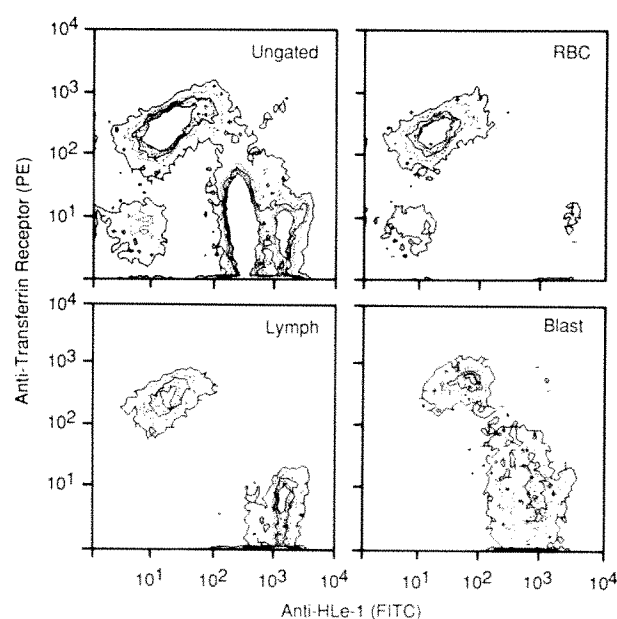


Fig 5. Correlation between transferrin receptor and HLe-1 expression on normal bone marrow gated by light-scattering characteristics. Transferrin receptor and glycophorin expression were very similar when correlated with HLe-1 (as compared with Fig 3). The cells that expressed neither transferrin receptor nor HLe-1 were within the RBC light-scattering window. A positive correlation existed between transferrin receptor and HLe-1 expression, evident in the "ungated" panel.

sorted into two populations based on transferrin receptor expression (Table 2). Erythrocytes with polychromasia were more plentiful in the population that expressed transferrin receptor. The relative proportion of cells comprising these two populations varied from one sample preparation to another. Because mature erythrocytes lacked transferrin receptor, the contamination of the marrow sample with mature blood cells increased the population that expressed glycophorin but not transferrin receptor. Sorting experiments also showed that all the glycophorin⁺, transferrin receptor⁺ cells in the lymph window were predominantly nucleated erythroid precursors (Table 2). In comparison, the transferrin receptor⁻, glycophorin⁻ cells were almost exclusively lymphocytes.

The complex correlation between transferrin receptor and glycophorin expression on cells in the blast window is also shown in Fig 6. There were cells that expressed high levels of transferrin receptor and glycophorin on their cell surfaces, cells that expressed neither antigen, and a continuum of cells that connected these two populations. The cells in this window were arbitrarily classified into groups as shown by the boxes in Fig 6. Table 3 presents the proportion of the total marrow cells in each of these populations and the morphologies of these subsets. The cells that were most strongly positive for both glycophorin and transferrin receptor (Fig 6, blast region A) were exclusively of the erythroid lineage and were mostly normoblasts. The cells with intermediate amounts of glycophorin and transferrin receptor (Fig 6, blast region B) were identifiable erythroblasts. The cells that had slightly lower quantities of transferrin receptor but no glycophorin (Fig 6, blast region C) also contained mainly erythro-

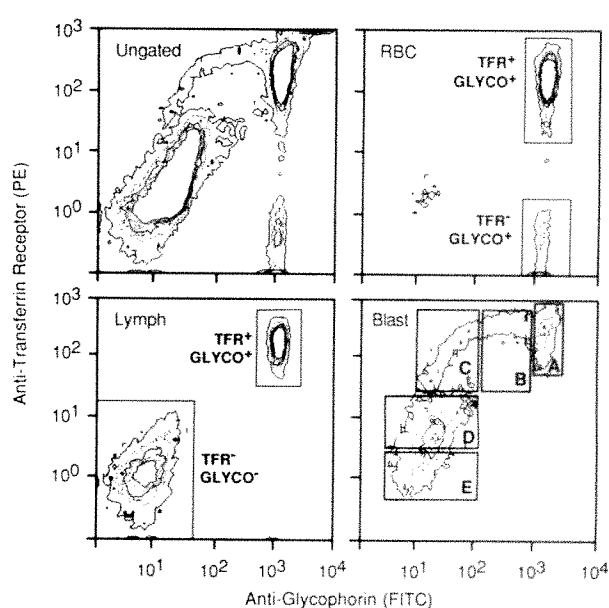


Fig 6. Correlation between transferrin receptor and glycophorin expression on normal bone marrow. Two populations of glycophorin⁺ cells could be identified in the RBC window based on transferrin receptor expression. These two populations as well as the two mutually exclusive populations identified in the lymph window were sorted for morphological examination (Table 2). The glycophorin and transferrin receptor expression of the cells within the "blast" light-scattering window showed a convex shape. Cells were sorted from each of the five boxes for morphological examination (Table 3), and for the enumeration of progenitor cells (Table 4).

Table 2. Differential Morphological Counts of Normal Human Bone Marrow Sorted by Light Scattering, Transferrin Receptor, and Glycophorin Expression

Windows*	RBC		Lymph	
	TFR - Glyco +	TFR + Glyco +	TFR - Glyco -	TFR + Glyco +
Antigen expression†				
Proportion in window‡	17	3	15	1
Cell type§				
Mature erythrocyte	80	52	1	2
Erythrocyte with polychromasia	20	42	0	18
Orthochromatic normoblast	0	6	0	60
Polychromatophilic normoblast	0	0	0	14
Basophilic normoblast	0	0	0	5
Erythroblast	0	0	0	1
Small lymphocyte	0	0	37	1
Large lymphoid	0	0	62	0

TFR, transferrin receptor; Glyco, glycophorin; Lymph, lymphoid cells.

*Normal human bone marrow cells stained with antitransferrin receptor and antiglycophorin were sorted based on both light-scattering properties and immunofluorescence. The light-scattering windows, RBC, and lymph, are illustrated in Fig 1.

†The immunofluorescence criteria used to sort the cells are shown in the boxes illustrated in Fig 6.

‡The proportion of cells classified within the sorting windows is expressed as a percentage of all cells.

§Cytocentrifuge slides were prepared from the sorted cell fractions and differential morphological counts were made after Wright's staining.

Table 3. Differential Morphological Counts of Blast Cells From Normal Human Bone Marrow Sorted by Light-Scattering, Transferrin Receptor, and Glycophorin Expression

Windows*	A	B	C	D + E
Proportion in window†	3.2	0.8	0.5	4.8
Cell type‡				
Erythrocyte	10	3	5	2
Orthochromatic normoblast	20	0	0	1
Polychromatophilic normoblast	29	0	0	2
Basophilic normoblast	10	0	0	0
Erythroblast	30	93	82	9
Undifferentiated blast	0	0	10	0
Small lymphocyte	0	0	0	2
Large lymphocyte	0	0	0	7
Plasma cell	0	0	0	4
Monocyte	1	0	0	34
Mono/Myeloblast	0	4	3	25
Band	0	0	0	5
Metamyelocyte	0	0	0	4
Myelocyte	0	0	0	4
Basophilic myelocyte	0	0	0	1

*Normal human bone marrow cells stained with both antitransferrin receptor and antiglycophorin were sorted based on both light-scattering and immunofluorescence properties. Blast cells, as illustrated in Fig 1, were classified into four groups based on immunofluorescence staining as shown in the boxes in Fig 6. For the morphological studies, the cells in boxes D and E were combined.

†The proportion of cells classified in the sorting windows is expressed as a percentage of total cells.

‡Cytocentrifuge slides were prepared from the sorted fractions and differential counts were made of the Wright's-stained slides.

blasts, but in addition included morphologically "undifferentiated" blasts with large distinctive myeloperoxidase-positive red granules. The cells that were negative for both transferrin receptor and glycophorin (Fig 6, blast regions D, E) were mostly blasts, predominantly of the granulocytic and monocytic lineages.

The populations identified in Fig 6 were sorted and tested for their ability to form colonies in vitro (Table 4). Cells that were bright for both transferrin receptor and glycophorin could not form erythroid colonies (Table 4, window A). Cells within the other windows contained erythroid colony-form-

ing cells with different degrees of enrichment. The greatest enrichment for erythroid colonies on a per-cell basis was found in windows B or C depending on the experiment. Between 20% and 30% of the cells sorted could form erythroid colonies. The majority of erythroid colony-forming cells were found in window D, however. These cells had slightly elevated amounts of transferrin receptor but did not express glycophorin. The granulocyte-macrophage colonies were found almost exclusively in windows D and E in two experiments, but were found in windows B through C in a third experiment. Surprisingly, CFU-E were not clearly separated from BFU-E by transferrin receptor and glycophorin expression. These two colony-forming cell types, operationally defined in these experiments by colony morphology at day 8 v day 14 of culture, were therefore relatively heterogeneous in their transferrin receptor and glycophorin levels.

Nucleic acid content of erythroid cells. The last stages of erythroid development are characterized by the loss of the nucleus and subsequent loss of RNA-containing reticulum. These steps were identified by quantifying the nucleic acid content of the cells using propidium iodide (PI), which intercalates with both double-stranded DNA and double-stranded RNA.^{26,27} To use this nucleic acid stain, bone marrow cells were first labeled with fluorescent monoclonal antibodies and were then fixed with paraformaldehyde. After the cell membranes were permeabilized with detergent, the cells were stained for nucleic acid content.

The correlations between PI staining (on a logarithmic scale) and immunofluorescence, identified by either antitransferrin receptor or anti-HLe-1, are shown in Fig 7. Three cell populations were identified by their different intensities of PI staining in Fig 7A, when an irrelevant (goat anti-mouse IgG FITC) antibody was used. The transferrin receptor⁺ cells (Fig 7B), included a strongly PI⁺ population and a population with intermediate intensity of PI staining. This population of cells with intermediate staining of PI expressed low amounts of HLe-1 (Fig 7C). This intermediate amount of PI staining was shown to be a result of double-stranded RNA (rather than DNA) by the loss of staining after these cells were treated with ribonuclease A (Fig 7D). These reticulocytes were distinguished from platelets, which

Table 4. Progenitor Cell Assays of Normal Human Bone Marrow Cells Sorted by Light Scattering, Transferrin Receptor, and Glycophorin Expression

Experiment*	I			II					III					IV				
Window†	A	B/C	D/E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Proportion in window‡	1.5	2.0	7.4	1.8	0.2	0.2	4.2	1.5	0.7	0.2	0.4	4.4	2.1	—§	0.2	0.3	2.8	1.1
CFU-E	0	343	115	0	10	350	90	29	3	0	585	172	79	—	749	552	271	13
BFU-E	0	14	8	0	0	28	7	4	0	0	35	20	11	—	100	82	0	0
CFU-C	0	38	79	—¶	—	—	—	—	0	0	0	75	155	—	0	2	132	29

*Four replicate experiments were completed.

†Normal bone marrow cells stained with antitransferrin receptor and antiglycophorin were sorted based on both light-scattering and immunofluorescence properties. The blast cells, Fig 1, were divided into five fractions based on glycophorin and transferrin receptor expression as shown in the boxes in Fig 6. In experiment I, cells in boxes B and C as well as D and E were combined and assayed together.

‡The proportion of cells classified into the sorting windows are expressed as a percentage of total cells.

§In experiment IV, fraction A was not assayed.

||The progenitor cells were assayed as described in the Materials and Methods section. The number of colonies is expressed per 2,000 cells sorted.

¶In experiment II, the cells were not assayed for CFU-C.

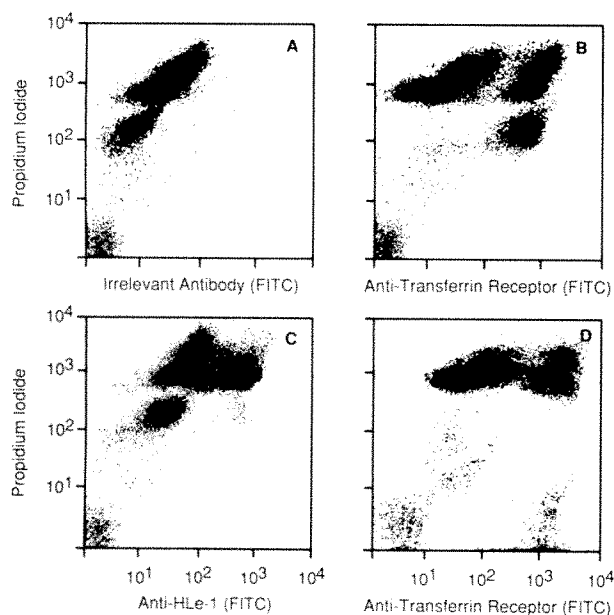


Fig 7. Discrimination of marrow populations based on nucleic acid content. Bone marrow cells were reacted with antitransferrin receptor FITC (B,D), anti-HLe-1 FITC (C), or with an irrelevant antibody (goat anti-mouse FITC, A). These populations were then stained with propidium iodide (PI) as described in the Materials and Methods section. The cells in panel D were also treated with ribonuclease A. The population of cells with intermediate PI staining were brightly reactive with antitransferrin receptor but were unlabeled with anti-HLe-1. This intermediate PI staining was sensitive to ribonuclease, as shown in panel D.

also contain RNA, since the erythroid cells expressed glycophorin whereas the platelets did not (data not shown).

DISCUSSION

The continuous changes in characteristics associated with the development of hematopoietic cells can be demonstrated by quantitative multiparameter analysis. The correlations among three cell surface markers (glycophorin A, transferrin receptor, and HLe-1), physical characteristics, and nucleic acid content defined the erythroid lineage and its developmental stages. A summary of the data presented in this article is shown in Fig 8, in which the relative quantities of particular antigens are correlated with other changes characterizing erythroid maturation. This approach required multiple-parameter analysis and a large, dynamic range of quantitative detection, since the data are spread over four decades of intensity.

Glycophorin expression was confirmed as a feature of the later stages of erythroid development (glycophorin was not expressed on erythroid colony-forming cells; Table 4). Glycophorin appeared at the erythroblast stage and rapidly reached maximum expression by the normoblast stage. Glycophorin expression was especially useful to discriminate the later erythroid cells from lymphoid cells, since cells of both of these lineages have similar light-scattering properties (Fig 2). Once glycophorin was maximally expressed, the amount in each erythroid cell was remarkably constant from cell to cell and did not change during further maturation (Figs 3

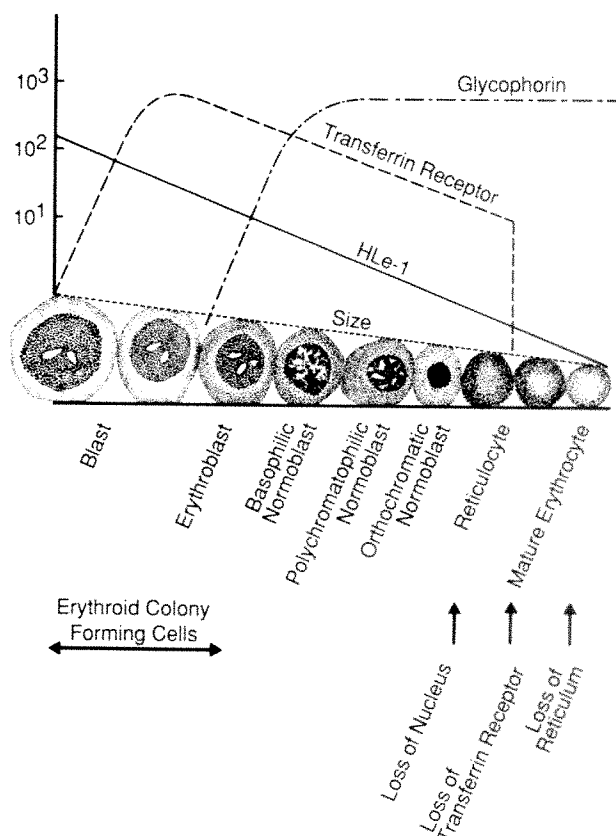


Fig 8. Schematic representation summarizing the characteristics of erythroid cells as they mature. The relative quantities of the cell surface antigens are depicted in the upper portion of the diagram. These antigenic characteristics can be correlated with cell size, morphology, nucleic acid content, and erythroid colony-forming ability.

and 6), even though erythroid cell size decreased during maturation.²⁸ This suggested a net increase in glycophorin density on the cell surface.

The expression of transferrin receptors on erythroid cells preceded the expression of glycophorin. (This was indicated in the convex shape of the bivariate correlation between transferrin receptor and glycophorin shown in Fig 6.) Most erythroid colony-forming cells expressed low to intermediate amounts of transferrin receptor. Unexpectedly, we could not separate CFU-E and BFU-E into discrete fractions based on transferrin receptor/glycophorin levels, although CFU-C tended to have lower expression of transferrin receptor than did BFU-E and CFU-E. The observations by us and others that some erythroid colony-forming cells are apparently transferrin receptor⁻ may be accounted for by quantitative, rather than qualitative, differences.^{1,11} Expression of transferrin receptor is nearly maximal before glycophorin is expressed. Once erythroid cells expressed cell surface glycophorin, they were no longer able to form colonies.⁸ Erythroid lineage cells expressed much higher maximal levels of cell surface transferrin receptor than did cells of other lineages (Fig 5). In addition, transferrin receptor persisted to a very late stage of erythroid cell development (reticulocyte). Both these observations may be related to the unique requirement

of erythroid cells for large quantities of iron needed for hemoglobin synthesis. This quantitative difference in transferrin receptor expression, as well as the decrease in HLe-1 (Fig 5), facilitated the identification of early erythroid cells as distinct from other lineages of cells. After the transferrin receptor reached maximal levels on the erythroid cell surface, its expression decreased progressively with further erythroid maturation, with a dramatic loss of antigen at the late reticulocyte stage (as defined by loss of detectable RNA).

There was a parallel decrease in HLe-1 and transferrin receptor levels during erythroid maturation (Fig 5). The loss of HLe-1 during the development of erythroid cells began almost as soon as the cell became committed to the erythroid lineage. The decreased expression of the T200, common leukocyte antigen (HLe-1) and the increased expression of the transferrin receptor were very early events following the commitment of cells to the erythroid lineage.

The physical characteristics of cells identified by light scattering also changed gradually during the development of erythroid cells. Cell size, as detected by forward-angle light scatter, decreased as the erythroid cells matured (Tables 1 and 2). No abrupt change in forward light-scattering occurred when erythroid cells lost their nuclei. After enucleation, right-angle light scatter increased; this may have resulted from the acquisition of biconcave disc morphology. This increase in right-angle light scattering was seen only on viable cells. (After paraformaldehyde fixation, mature erythrocytes and lymphoid cells had indistinguishable light-scattering properties—data not shown.) Although erythroid and lymphoid cells had similar light-scattering characteristics, they were clearly distinguished by their cell surface antigenic markers.

PI staining was used to measure nucleic acid content of erythroid cells. The loss of the nucleus from the transferrin receptor⁺, glycophorin⁺ normoblast was easily identified by the decrease in PI fluorescence. After the loss of the nucleus, the cells still contained RNA and retained the same cell surface phenotype (transferrin receptor⁺, glycophorin⁺, and HLe-1⁻). These operationally defined reticulocytes next lost transferrin receptor, then the remainder of their RNA, to become mature erythrocytes (transferrin receptor⁻, glycophorin⁺, HLe-1⁻).

The discrete population of cells identified as reticulocytes (Fig 7) may be an artifact resulting from the Ficoll-Hypaque preparation of the bone marrow. Others have shown a continuous loss of RNA during reticulocyte maturation, which is accompanied by a change in buoyant density of the cells.³¹ Therefore, one would expect the very earliest reticulocytes to be found in the buoyant fraction of the density gradient, whereas the later reticulocytes would sediment with the mature erythrocytes. This would result in the identification of a discrete population of early reticulocytes.

The correlated expression of multiple cellular characteristics provided a basis for study of the progression of cells through a single lineage from the most immature to the mature cells. It will be interesting to add other markers, which further subdivide the development of the erythroid lineage, to define more precisely the progression and timing of cellular events during erythroid maturation. By identifying an entire lineage of cells, as we have done with the erythroid lineage, it is also possible to eliminate these cells from the study of other lineages. Thus, it is potentially possible to define precisely the maturational sequence of each lineage of cells by taking advantage of the appropriate cell surface, physical, and biochemical properties.

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Heterogeneous Distribution of Transferrin Receptors on Parenchymal and Nonparenchymal Liver Cells: Biochemical and Morphological Evidence

By Wolfgang Vogel, Adrian Bomford, Stephen Young, and Roger Williams

To investigate which cells of the liver express the receptor for transferrin, isolated rat liver cells produced by collagenase perfusion were fractionated by repeated differential centrifugation to produce hepatocytes (95% + 1%, mean $\pm$ SD, $n = 4$) and nonparenchymal cells (97% + 1%, $n = 3$). Saturable, high-affinity binding of ^{125}I -transferrin was demonstrated on intact cells at 4 °C, with average receptor numbers $20,900 \pm 3,160$ (mean + SD, $n = 4$) for hepatocytes and $5,500 \pm 1,520$ ($n = 3$) for nonparenchymal cells. Total cellular receptors measured in detergent permeabilized hepatocytes were $42,000 \pm 18,330$ (mean $\pm$ SD, $n = 3$) per cell and $14,760 \pm 7,120$ ($n = 3$) per cell in the nonparenchymal fraction. Immunocytochemical demon-

stration of transferrin using antitransferrin, peroxidase antiperoxidase complex confirmed that both cell types bound transferrin. There was heterogeneity of the staining reaction since there was no detectable staining on 40% of hepatocytes and 60% of nonparenchymal cells. Microdensitometric analysis of the staining product corroborated the biochemical evidence that hepatocytes have, on average, more than three times more transferrin receptors than do nonparenchymal cells. These findings support the concept that the hepatocyte has a central role in the uptake and storage of transferrin iron.

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THE LIVER HAS a central role in mammalian iron metabolism, containing one-third of total body iron.¹ This is stored, mainly in the form of ferritin, in the hepatocytes and to a lesser extent in the nonparenchymal cells of the liver,² namely Kupffer and endothelial cells. On the basis of biochemical evidence, it has been suggested that the uptake of iron into this store is mediated through the presence of a specific saturable binding site on the hepatocyte, the receptor for transferrin.³⁻⁵ The histochemical demonstration of transferrin receptors, by use of a monoclonal antibody on both parenchymal and nonparenchymal cells in human liver tissue, supports this finding.⁶ In direct contrast to the above, however, Soda and Tavassoli and Kishimoto^{7,8} recently reported that they could demonstrate neither binding of transferrin-coated minibeads to parenchymal cells in rat liver by electron microscopy nor binding of radiolabeled transferrin at 4 °C. They concluded that only hepatic endothelial cells express transferrin receptors.

This claim has led us to reexamine the question of which cells in the liver express receptors for transferrin. In the present study, we paid particular attention to purification of hepatocytes from nonparenchymal cells in preparations of rat liver cells obtained by collagenase perfusion.⁹ We present biochemical and morphological evidence that both rat hepatocytes and nonparenchymal liver cells express the receptor for transferrin.

MATERIALS AND METHODS

Cell preparations. Liver cells were obtained from male Wistar rats (200 to 250 g) using Berry and Friend's collagenase (type IV, Sigma, Poole, UK) perfusion method⁹ modified as described.⁴ After filtering through a nylon mesh, the total cell suspension was immedi-

ately transferred to 4 °C and was kept at this temperature during further processing. Siliconized glassware and prechilled plastic tubes were used. The total cell yield was between 650 and 720×10^6 cells per liver, and the viability was 98%, as judged by trypan blue exclusion. To prepare nonenzymatically separated hepatocytes, open fine-needle aspiration biopsies of livers¹⁰ in ether-anesthetized rats were performed. Approximately 10^5 hepatocytes were obtained from a biopsy and washed three times in Hanks' buffered salt-solution (HBSS) containing 1% (wt/vol) bovine serum albumin (BSA) at 37 °C. The cells were then spun onto slides and processed as described later for cells obtained by collagenase perfusion.

The total cell suspension from a collagenase-perfused liver was centrifuged at 50 g for 2 minutes in ice-cold HBSS containing 1% (wt/vol) BSA, and the supernatants were pooled.¹¹ The pellets were resuspended in wash buffer. These centrifugations were carried out up to 10 times. The final pellet was kept at 4 °C after resuspension. The collected supernatants were further centrifuged for 2 minutes at 50 g to remove the remaining hepatocytes, and the pellet obtained by centrifuging these at 700 g for 7 minutes was layered onto a discontinuous iso-osmotic Percoll (Pharmacia, Milton Keynes, Bucks, UK) gradient at a concentration of 10×10^6 cells/15 mL of gradient. The gradients were prepared by adding the required volume of phosphate-buffered saline (PBS), (285 mOsm l^{-1} pH 7.4) to give Percoll solutions of densities 1.08, 1.075, 1.065, 1.055, and 1.045 g/mL. After centrifugation at 800 g for 50 minutes at 4 °C in a MSE Mistral Coolspeed using a swing-out rotor, the fractions between 1.055 and 1.075 g/mL¹² were collected and washed three times in wash buffer by spinning at 500 g for 7 minutes.

Assessment of cellular composition. Aliquots (5×10^4 cells) of the well-mixed suspensions of purified cell preparations were spun onto slides after the separation process, using a Shandon Cytospin 2 at 350 rpm for 15 minutes, air-dried, fixed in cold acetone, and stained with hematoxylin. A total of 2,000 cells from high-power fields representing central and peripheral areas of two specimens of each fraction were examined on a Leitz 20 EB microscope fitted with eyepiece graticules. Hepatocytes and nonparenchymal cells could easily be distinguished by their typical size and morphology.¹³ Kupffer cells were identified by staining for their endogenous marker enzyme peroxidase¹⁴ according to Graham and Karnovsky.¹⁵

Proteins. Transferrin was purified from pooled human and pooled rat plasma to which ferrous ammonium sulphate was added to iron-load the transferrin. A modification of the method of Okada and colleagues¹⁶ was used, in which the ammonium sulphate fractions precipitated between 35% and 75% saturation were desalted by extensive dialysis against distilled water and chromatographed at 4 °C on a column (gel bed, 2×60 cm) of diethylaminoethyl

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(DEAE)-Sephacrose (Pharmacia), using a linear 50- to 350-mmol/L gradient of Tris/HCl, pH 8. The pink fractions were pooled, concentrated by ultrafiltration, and reappplied to the column using a 50- to 160-mmol/L Tris gradient as before. Fractions with A470/A410 ratios of >1.0 were pooled and rechromatographed to yield fractions of ratios of >1.3 . This procedure gave a single protein peak in the case of human transferrin and two distinct peaks with rat transferrin. These rat isotransferrins, previously characterized as Tf₁ and Tf₂,¹⁶ were present in a ratio of $\sim 2:1$, respectively. The major isotransferrin Tf₁ from three separate preparations of rat transferrin was used in the present study. These isotransferrins have been previously shown to donate iron equally to the liver and other tissues in the rat.¹⁷

The diferric transferrins were radiolabeled with ¹²⁵I (Amersham International, Bucks, UK) using solid-phase lactoperoxidase as previously described,¹⁸ and the iron saturation of the proteins was confirmed after iodination by polyacrylamide gel electrophoresis (PAGE) in 6 mol/L of urea.¹⁸ Specific activities of the labeled proteins ranged from 500 to 900 cpm/ng. To prepare ¹²⁵I-labeled apotransferrin, the labeled diferric species were dialyzed at 4 °C against three changes of 0.1 mol/L of sodium citrate/acetic acid, pH 4.5, followed by exhaustive dialysis against deionized double-distilled water.¹⁸

Binding assays. Binding assays on intact cells were performed by adding cells to HBSS 1% BSA containing ¹²⁵I-labeled transferrin in a final 0.5-mL vol in flat-bottomed polystyrene tubes. The tubes were gassed with 95% O₂/5% CO₂ and incubated with shaking for 90 minutes at 4 °C. Parallel incubations were performed with at least 100-fold excess of unlabeled human transferrin to estimate nonspecific binding. The cells were washed three times in ice-cold PBS by centrifuging at 500 g at 4 °C and were counted in a γ counter.

To determine the binding affinities of ¹²⁵I-labeled diferric human and rat transferrins to the rat transferrin receptor, rat reticulocytes were produced by phenylhydrazine injection¹⁹ and $2 \pm 0.5 \times 10^8$ washed reticulocytes were added to each tube; 0.1 to 20 μ g/mL of ¹²⁵I-labeled transferrin was added, as was unlabeled transferrin when indicated. In some experiments, ¹²⁵I-labeled rat and human apotransferrins were used. The binding buffers contained 100 μ mol/L of desferrioxamine (Ciba Pharmaceuticals, Horsham, Sussex, UK) to maintain the proteins in the iron-free form.¹⁸ Because apotransferrin binds with lower affinity to the transferrin receptor than does the diferric species, a higher concentration of the labeled proteins (1 to 200 μ g/mL) was used in the binding studies to ensure saturation of the receptor.¹⁸

The binding of diferric transferrin to liver cells was performed by incubating $1.8 \pm 1.0 \times 10^6$ nonparenchymal cells, or $2 \pm 0.25 \times 10^6$ parenchymal cells to each tube with 0.1 to 20 μ g/mL of ¹²⁵I-human transferrin. Alternatively, in experiments to evaluate receptor number in a competitive setting, a fixed amount ($\sim 2,500$ cpm) of ¹²⁵I-diferric human transferrin, labeled to high specific activity ($\sim 5,000$ cpm/ng) was added to each tube. An increasing amount of unlabeled human or rat transferrin (up to 0.5 mg/mL) was added, and the incubations were carried out at 4 °C for 90 minutes with shaking. Cells were washed in PBS and counted as before.

Total cellular receptor number (the sum of membrane and intracellular receptors), was determined on detergent-solubilized cell extracts.²⁰ Six to 12×10^6 cells were dissolved in 1.2 mL of PBS containing 0.1% (vol/vol) Triton X-100 (Sigma) and 1 mmol/L of phenylmethylsulfonyl fluoride (Sigma) and centrifuged at 12,000 g for 15 minutes at 4 °C. The supernatant was divided into 0.2-mL aliquots in 1.5-mL microcentrifuge tubes, and 20 μ g of unlabeled human transferrin was added to two of these. After 15 minutes at 22 °C, 0.3 mL of 0.1 mol/L of Tris/citrate pH 5, containing 0.1% (wt/vol) BSA, 0.1% (vol/vol) Triton X-100, and 200 ng of ¹²⁵I-human transferrin (diferric) was added to all tubes. These were

incubated for 30 minutes at 37 °C, and the transferrin-receptor complex was precipitated by the addition of 0.8 mL of polyethylene glycol 6,000 (12% wt/vol) in 0.1 mol/L of Tris/citrate pH 5.0 containing human γ -globulin (0.1% wt/vol) for 30 minutes at 4 °C. The tubes were centrifuged at 12,000 g for 15 minutes at 4 °C, and the supernatant and precipitate were counted in a γ counter.

Immunoperoxidase technique. Cytospin monolayer cell preparations of the mixed postperfusion population and purified preparations of parenchymal and nonparenchymal cells were air-dried, fixed in cold acetone for 20 seconds,²¹ and further processed as described by Faulk and co-workers,²² modified for the indirect immunoperoxidase method at 4 °C. Endogenous peroxidase was blocked by incubation with 1.5% H₂O₂-PBS (vol/vol) for 30 minutes; incubation with transferrin (0.53 mg/mL) was carried out for 30 minutes, followed by two washes in PBS for 15 minutes. To prevent nonspecific background staining, the specimens were then incubated with 20% normal swine serum (Dakopatts, High Wycombe, Bucks, UK) for 10 minutes. Because human transferrin binds to the rat transferrin receptor with higher affinity than does the native protein (described in Results section), we used human transferrin and a specific heat-inactivated (30 minutes at 56 °C) antiserum against human transferrin raised in rabbits²³ at the highest possible dilution. Radial immunodiffusion and immunoelectrophoresis²⁴ indicated that this antiserum was specific for human transferrin and failed to react with rat transferrin. Control incubations were performed with: (a) purified rat transferrin as ligand, (b) with human apotransferrin in PBS containing 100 μ mol/L of desferrioxamine¹⁸ (Ciba) as ligand, (c) without transferrin, and (d) without antibody. Commercially available bridging antibody and peroxidase-antiperoxidase (PAP)-complex were used in recommended dilutions (Dakopatts). The peroxidase reaction was performed with 0.5% (wt/vol) 3'-3'-DAB (Sigma) Tris-HCl pH 7.8 solution for 2 minutes at room temperature. H₂O₂ was added to a final concentration of 0.5% (vol/vol) just before use.

Microdensitometric quantitation. The colored product of the peroxidase reaction on a specimen containing the mixed parenchymal-nonparenchymal cell populations was quantitated with a Vickers M85 scanning and integrating microscope. Absorbance at 430 nm was measured with a $\times 40$ objective, spot size 1 (0.5 m in diameter in the optical plane of the specimen), field size of 25 m for parenchymal cells or 10 m for nonparenchymal cells. Under these conditions, single cells of randomly chosen clusters throughout the specimen were evaluated after the microscope was calibrated as described.^{25,26} The absorption expressed in arbitrary machine units was corrected for the slide-background, which was assessed by scanning a cell-free area of the specimen.

RESULTS

Comparison of binding of human and rat transferrins to the rat transferrin receptor. To distinguish between receptor-bound ligand and endogenous transferrin in the immunocytochemical staining of liver cells, we occupied the cellular receptors with human diferric transferrin and recognized this with a polyclonal anti-human transferrin antiserum that did not react detectably with rat transferrin. For this reason, experiments were performed first to characterize the binding of ¹²⁵I-human transferrin to the rat transferrin receptor and then to compare this with the binding of iodinated rat transferrin. Equilibrium binding experiments were performed at 4 °C using rat reticulocytes and ¹²⁵I-human diferric transferrin and ¹²⁵I-rat diferric transferrin as ligands. As shown in Fig 1, in which the data from a representative experiment have been plotted according to the method of Scatchard,²⁷ human transferrin (K_d 4.7×10^8 l mol⁻¹) bound

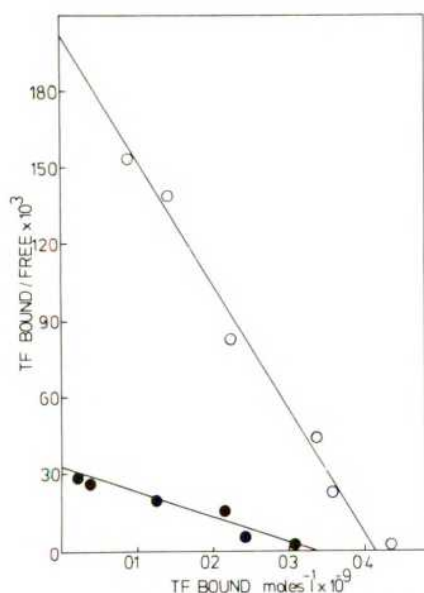


Fig 1. Comparison of the binding of human and rat transferrin to rat reticulocytes. Rat reticulocytes were produced by phenylhydrazine injection and incubated (2×10^8 cells/0.5 mL of Hanks' Balanced Salt Solution, 1% bovine serum albumin) with up to 10 $\mu\text{g/mL}$ of ^{125}I -labeled human or rat transferrin for 90 minutes at 4°C in air, 5% CO_2 . Non-specific binding was estimated in the presence of 100-fold excess of unlabeled human or rat transferrin, respectively. Cells were washed three times in phosphate-buffered saline (PBS) at 4°C and counted in a γ counter. The binding data for human transferrin (O) K_d , $4.6 \times 10^8 \text{ l/mol}^{-1}$; and rat transferrin (●) K_d , $1 \times 10^8 \text{ l/mol}^{-1}$ were plotted according to the method of Scatchard.²⁷

to the rat transferrin receptor with an affinity nearly 5 times higher than that of the homologous transferrin (K_d $1 \times 10^8 \text{ l/mol}^{-1}$). Similar numbers of receptors were recognized by the two transferrin species. Three preparations of human transferrin purified from different batches of serum bound with consistently higher affinity (K_d $6.5 \pm 2 \times 10^8 \text{ l/mol}^{-1}$, mean $\pm$ SD) than that of three preparations of rat transferrin (K_d $0.9 \pm 0.4 \times 10^8 \text{ l/mol}^{-1}$). Confirmation of this difference in the affinity of binding was obtained from experiments in which a fixed amount of ^{125}I -human diferric transferrin was added to rat reticulocytes, and unlabeled human or rat transferrin was used to compete for binding. Fifty percent inhibition of binding was achieved by concentrations of $6.9 \pm 2.6 \times 10^{-8} \text{ mol/L}$ and $4.3 \pm 2.8 \times 10^{-7} \text{ mol/L}$ (mean $\pm$ SD, $n = 3$) with human and rat transferrin, respectively.

The differential in binding affinity was not present, however, in the case of the iron-free proteins, since both human (K_d $2.9 \pm 3.3 \times 10^8 \text{ l/mol}^{-1}$; mean $\pm$ SD, $n = 3$) and rat (K_d $2.5 \pm 2.4 \times 10^8 \text{ l/mol}^{-1}$) apotransferrins demonstrated very low affinity for the receptor.

Cell purification. The total yield of hepatocytes obtained by collagenase perfusion was reduced by differential centrifugation to $30\% \pm 6\%$ (mean $\pm$ SD, $n = 3$). Recovery of nonparenchymal cells from the Percoll gradients after three washings varied between 30% and 50% of the cell number applied. The initial cell viability of $>98\%$ was decreased slightly by the separation procedure but remained

at 95%. The purity of the cell suspension used for the receptor assays was $95\% \pm 1\%$ (mean $\pm$ SD, $n = 4$) for hepatocytes and $97\% \pm 1\%$ (mean $\pm$ SD, $n = 3$) for nonparenchymal cells. This latter fraction contained $30\% \pm 3\%$ (mean $\pm$ SD, $n = 3$) mononuclear cells positive for endogenous peroxidase.

Immunocytochemical demonstration of transferrin binding. In all preparations of liver cells, human transferrin had bound to hepatocytes and nonparenchymal cells, as shown by the antibody-mediated PAP staining of both types of cell (Fig 2). The staining product on the hepatocytes (H in Fig 2) and nonparenchymal cells (NP in Fig 2) was granular and distributed over the cells. The staining reaction was regarded as specific since (a) preincubation of the specimen with purified rat transferrin, (b) omission of the human transferrin, or (c) omission of antibody to human transferrin abolished the staining. Furthermore, when human apotransferrin was used as ligand, under conditions to ensure that it remained iron-free,¹⁸ staining was also abolished. Figure 2 shows that there was individual variation in the staining of the hepatocytes since the cells in the lower part of the Fig 2 showed granular staining whereas those in the upper part demonstrated minimal staining. This heterogeneity was further investigated by staining preparations of purified hepatocytes and nonparenchymal cells. As shown in detail in the following section, 60% of hepatocytes (Fig 3a) showed granular staining of variable intensity, with the remaining cells showing diffuse, very weak staining. In nonparenchymal cells, staining was seen on 30% to 40% of the cells, with the remaining cells showing very weak staining (Fig 3b). This heterogeneity of staining of hepatocytes was also seen on cells that had not been exposed to collagenase and that had been produced by fine-needle liver biopsy. Figure 4 shows two views of such a preparation.

Microdensitometry. To provide an objective comparison of the intensity of the color products on nonparenchymal cells

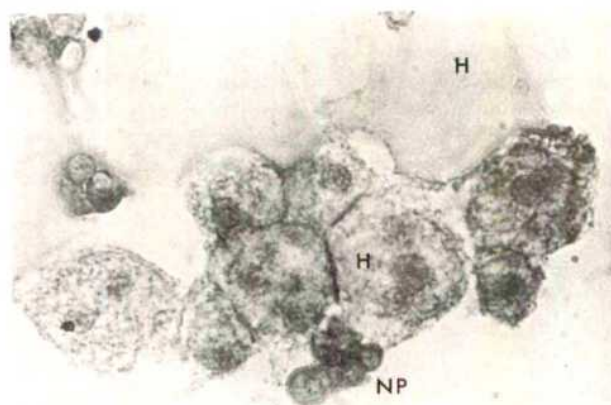


Fig 2. Anti-human transferrin antibody peroxidase-anti-peroxidase (PAP)-stained preparation of mixed rat liver cells (postperfusion). Hepatocytes (H) and nonparenchymal cells (NP) can easily be identified by their different size and shape. Staining reaction varies between neighboring hepatocytes, suggesting differences in transferrin binding. Leitz photomicroscope, $\times 40$ objective, Ilford Pan F, original magnification; current magnification $\times 19$.

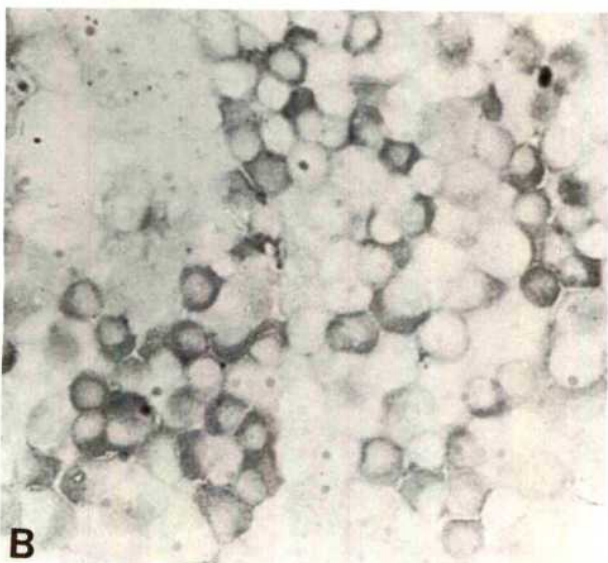
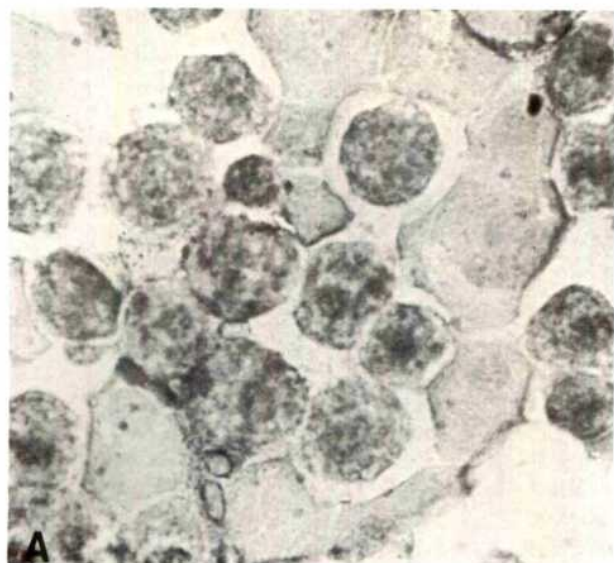


Fig 3. Anti-human transferrin antibody peroxidase-anti-peroxidase (PAP)-stained preparations for purified (A) hepatocytes and (B) nonparenchymal cells. Hepatocytes and nonparenchymal cells show individual variation in staining intensity, with a subgroup of both cell types showing minimal staining.

($n = 380$) and hepatocytes ($n = 350$), a preparation of mixed cells was scanned using a microdensitometer. An estimate of the absorbance due to nonspecific staining and light scattering was obtained by evaluating 20 cells of each type on a control slide from which the antitransferrin antibody had been omitted during the processing. As shown in the histogram (Fig 5) 150 nonparenchymal cells and 205 hepatocytes gave absorbance values greater than those obtained from cells on the control slide. In the nonparenchymal cells, values ranged from 5 to 35 machine units, whereas values in the hepatocytes ranged from 15 to 130 machine units. Comparison of the range of absorbance for the two groups using the Mann-Whitney test²⁸ showed that they were significantly different ($P < .001$).

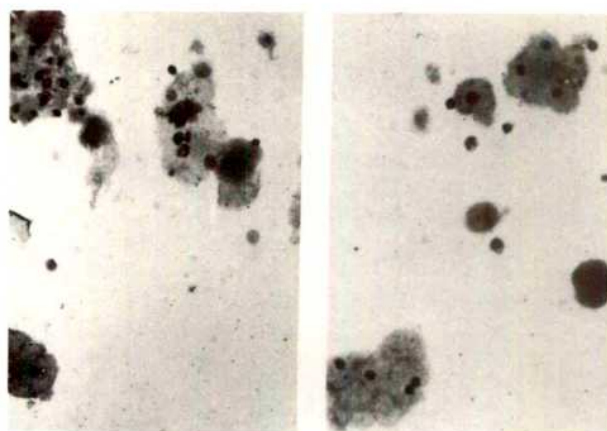


Fig 4. Anti-human transferrin antibody peroxidase-anti-peroxidase (PAP)-stained preparation (two views) of hepatocytes not exposed to collagenase and produced by fine-needle liver biopsy. Heterogeneity of staining intensity of individual cells is seen (original magnification $\times 25$; current magnification $\times 12$).

Binding of ^{125} I-human transferrin to hepatocytes and nonparenchymal cells. Because of the evidence that dimeric human transferrin binds with high affinity to the rat transferrin receptor, this ligand was used in a series of experiments to investigate the binding of transferrin to purified preparations of hepatocytes and nonparenchymal cells. There was saturable binding of ^{125} I-transferrin at 4 °C to cells of both fractions (Fig 6) with saturation occurring at >12.5 nmol/L of transferrin. Scatchard analysis²⁷ of the data (Fig 7) showed that ^{125} I-transferrin bound to $20,900 \pm 3,160$ (mean $\pm$ SD, $n = 4$) sites per cell on parenchymal cells with a K_d of $2.6 \pm 0.8 \times 10^8$ l mol⁻¹ (mean $\pm$ SD, $n = 4$). In the nonparenchymal cell fraction transferrin bound to $5,500 \pm 1,520$ (mean $\pm$ SD, $n = 3$) sites per cell with a K_d of

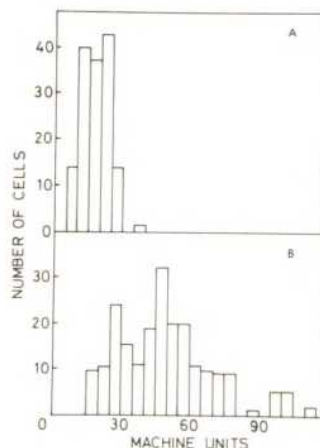


Fig 5. Frequency diagram of the microdensitometric absorbance (430 nm) of anti-human transferrin antibody peroxidase-anti-peroxidase (PAP) on random clusters of (A) nonparenchymal cells and (B) hepatocytes; 380 nonparenchymal cells and 350 hepatocytes were scanned. When the absorbance values were corrected for nonspecific staining, 40% of the hepatocytes and 60% of the nonparenchymal cells gave no measurable absorbance. Shown are the values for the remaining cells giving detectable absorbance.

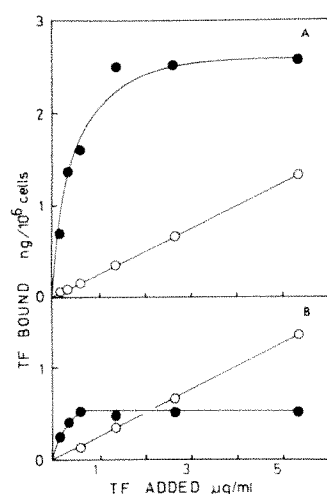


Fig 6. Concentration-dependent binding of human ^{125}I -transferrin to purified (A) hepatocytes and (B) nonparenchymal cells at 4°C . Up to 3×10^6 cells were incubated in 0.5 mL of Hanks' balanced salt solution, 1% bovine serum albumin with 0.1 to 20 $\mu\text{g}/\text{mL}$ of transferrin for 90 minutes under air, 5% CO_2 . Cells were washed three times in phosphate-buffered saline at 4°C and counted in a γ counter. Specific binding ($\bullet$) was calculated by correcting total binding for nonspecifically bound counts ($\circ$) determined in the presence of 100-fold excess of unlabeled transferrin.

$3.3 \pm 1.9 \times 10^8 \text{ l mol}^{-1}$ (mean $\pm$ SD, $n = 3$). At saturation, nonspecific binding was 15% of the total binding in the parenchymal cell fraction and 20% of that in the nonparenchymal cell fraction. To investigate transferrin binding sites in a competitive setting, ^{125}I -human transferrin was added to purified parenchymal and nonparenchymal cells and either unlabeled human or rat transferrin was used to compete for binding. As shown in Table 1, similar numbers of binding sites were detected using this procedure as compared with the method used in Fig 7, with approximately four times more binding sites demonstrated on parenchymal cells than on nonparenchymal cells.

The above studies using intact cells at 4°C provide an estimate of the number of transferrin binding sites present on the external plasma membrane. To estimate the total number of receptors, assays were performed on extracts of cells

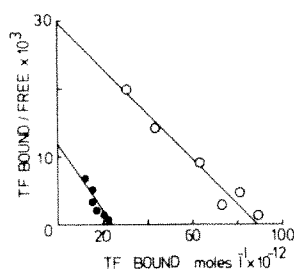


Fig 7. Scatchard²⁷ analysis of the binding data shown in Fig 6. In the experiment shown, the slope (K_d) for the hepatocytes ($\circ$) is equal to $3.4 \times 10^8 \text{ l mol}^{-1}$, and the value of the intercept with the abscissa indicates that 16.2×10^3 molecules of transferrin were associated with each cell. For the nonparenchymal liver cells ($\bullet$) the slope is $5.1 \times 10^8 \text{ l mol}^{-1}$ and the number of molecules bound was 3.8×10^3 per cell.

Table 1. Transferrin Binding Sites on Intact Cells at 4°C

Competing Ligand*	Parenchymal	Nonparenchymal
Human	$14,300 \pm 6,700$	$3,560 \pm 1,820$
Rat	$22,100 \pm 4,320$	$4,700 \pm 1,760$

Data are expressed as mean $\pm$ SD binding sites per cell. Cells were derived from three perfusions and fractionated by differential centrifugation as described in the Materials and Methods section.

*A fixed amount of ^{125}I -diferric human transferrin was added, and unlabeled transferrin (0 to 0.5 mg/mL) was added to compete for binding.

that had been solubilized in a detergent buffer. This treatment permeabilizes the cells and renders intracellular receptors available for binding as well as those present on the cell membrane.²⁰ With this technique, transferrin bound to $42,000 \pm 18,330$ (mean $\pm$ SD, $n = 3$) sites per cell in the parenchymal fraction and $14,760 \pm 7,120$ ($n = 3$) sites per cell in the nonparenchymal fraction.

DISCUSSION

The use of differential centrifugation¹¹ and colloidal silica (Percoll) gradient centrifugation¹² enabled us to prepare highly purified preparations of parenchymal and nonparenchymal cells from rat liver perfusates. This treatment is unlikely to have altered the characteristics of the cells significantly since they retained high viability ($> 90\%$) in both fractions and previous work²⁹ has shown that Percoll does not change the surface properties of isolated liver cells.

The hepatocyte is well established as the major site of transferrin synthesis³⁰, to avoid cross-reaction between endogenous transferrin and the antitransferrin antibody³¹ used during the immunocytochemical localization of receptor-bound ligand, we used human transferrin to occupy the receptors and its antibody, which did not react detectably with rat transferrin. Evidence for the specificity of this system came from the finding that staining could be abolished by occupying the receptors with rat transferrin and also by using human apotransferrin as ligand since at physiologic pH the iron-free form of transferrin has low affinity for its receptor.^{8,32} Because of this dependence on human transferrin, experiments were performed to characterize and compare the binding of human and of rat transferrin to the receptor on rat reticulocytes. Equilibrium binding experiments indicated that diferric human transferrin bound with high affinity to the rat receptor; furthermore, it bound with higher affinity than did rat transferrin. This difference in affinity was confirmed by the finding of at least a tenfold difference in the concentration of human ($6.9 \times 10^{-8} \text{ mol/L}$) and rat ($4.3 \times 10^{-7} \text{ mol/L}$) diferric transferrin required to displace 50% of ^{125}I -labeled human transferrin from rat reticulocytes. In contrast, both human and rat apotransferrins bound with an affinity ~ 1 order of magnitude less than the diferric proteins. This finding is in agreement with previous data showing very low binding affinity between the homologous apotransferrin and rabbit reticulocytes¹⁸ and rat liver cells.⁴

The present study provides morphological and biochemical evidence that human transferrin bound to receptors on

parenchymal as well as on nonparenchymal cells of rat liver. Intensity of staining showed individual variation, however, due to the specific binding of transferrin, as assessed visually and by microdensitometry, suggesting that some hepatocytes could express several times more receptors than the average number found using the radio-binding assays. The average of 20,000 surface receptors is in agreement with a previous report from this laboratory¹⁹ in which hepatocytes that had not undergone extensive purification to rid the parenchymal cells of contaminating nonparenchymal cells were used and rat transferrin was the ligand. The amount of transferrin bound in the present study, in which the titrations were done at 4 °C, was ~50% that reported previously for rat hepatocytes³⁻⁵ in which the analyses were performed at 37 °C. At the lower temperature, only surface receptors are occupied by ligand, whereas at 37 °C, endocytosis occurs and the full cellular complement of receptors is occupied.³³ Confirmation of the presence of a large intracellular pool of transferrin receptors in hepatocytes, as has been described for other types of receptor such as that for asialoglycoproteins,³⁴ was obtained from binding assays performed on permeabilized cells. This method showed that the total receptor number was >40,000 per cell.

Approximately 35% of the cells in the nonparenchymal fraction were peroxidase positive (Kupffer cells), a proportion similar to that observed in the intact liver,¹⁴ and it is likely that the separation techniques used here produced a representative population of nonparenchymal cells. Although we cannot comment at this stage on the distribution of transferrin receptors on different subpopulations of nonparenchymal cells, it is of interest that ~60% of these cells showed negligible binding of transferrin, suggesting (as with the hepatocytes) that an individual cell could have more receptors than the average found on this mixed population (surface ~5,000; total ~15,000) using the biochemical assays.

Despite the differences in receptor numbers expressed by the parenchymal and nonparenchymal cells, both bound transferrin with high affinity and similar association constants, which suggests that the cells express the same type of receptor. The values for the association constants obtained fall within the range reported for a variety of cultured cell lines^{32,33} as well as normal cells.³⁵

The finding that parenchymal cells have approximately three times the number of surface and total receptors as nonparenchymal cells is corroborated by the immunocytochemical demonstration of the binding of transferrin to the two cell types. Although the technique of microdensitometry cannot be used for the absolute quantitation of ligand binding, a comparison of the median absorbance range of the color reaction suggests that approximately three times more transferrin was bound by hepatocytes than by nonparenchy-

mal cells. We interpret the data showing heterogeneity of the color reaction between individual hepatocytes as indicating differences in receptor densities and the absence of transferrin binding to a proportion of hepatocytes and nonparenchymal cells as indicating that some cells express so few receptors that they are not detected by the techniques used here. Although transferrin receptors are maximally expressed by proliferating cells,^{36,37} this is unlikely to be the cause of receptor heterogeneity among hepatocytes, because the proportion of cells in the liver undergoing cell division is very small.³⁸ The heterogeneity may reflect different roles in iron metabolism of hepatocytes from different parts of the lobule, as has been shown for other hepatic functions.³⁹ Despite a recent claim to the contrary,⁴⁰ selective proteolytic damage to the transferrin receptors is unlikely since the perfusion buffers included a trypsin inhibitor⁴ and heterogeneity of staining was also seen on mechanically separated hepatocytes that had not been exposed to proteases. A study of the distribution of ferritin among individual hepatocytes would be of interest since it has been shown recently that there is an inverse relationship between the concentration of ferritin protein and expression of transferrin receptors by cultured human cells.²⁰

Our results do not substantiate the claim of Kishimoto and Tavassoli that transferrin receptors are expressed only by the nonparenchymal and specifically the endothelial cells of rat liver. This conclusion was based on their finding that ¹²⁵I-rat transferrin-coated minibeads failed to bind to isolated rat hepatocytes but were apparently specifically bound by endothelial cells. The uptake of minibeads could be a reflection of the accessibility of transferrin receptors on the different cell types since the membrane of endothelial cells is smooth, permitting access of immobilized ligand to its receptor. This contrasts with the more irregular membrane of the hepatocytes and Kupffer cells, which could compromise accessibility of ligand immobilized on relatively large beads. As discussed above, more recent claims by those workers that surface transferrin receptors are damaged by collagenase⁴⁰ are also unlikely, as we found that receptors were detectable on hepatocytes isolated by collagenase perfusion as well as by mechanical means. The concentration of ¹²⁵I-transferrin required to achieve saturable binding to liver cells in the studies of Kishimoto and Tavassoli was in excess of 1 μmol/L, which is in contrast to the results of the present study and to the experience of many workers using crude suspensions of hepatocytes³⁻⁵ and cultured cells^{20,32,33} in which saturation was achieved in concentrations ~1 order of magnitude less.

In conclusion, our finding that hepatocytes express, on average, three to four times more receptors than do nonparenchymal cells supports the concept that the hepatocyte has a central role in the uptake and storage of transferrin iron.

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Human Acute Unclassified Leukemia With a Unique t(4;17) Chromosomal Translocation Expresses T Lymphoid and Myeloid Surface Antigens After In Vitro Culture

By Arnold Ganser, Gerhard Heil, Thomas Böhm, Claus R. Bartram, Anand Raghavachar, Felix Carbonell, and Dieter Hoelzer

Bilineage differentiation along both the T lymphoid and the myeloid lineage while in vivo diffusion chamber (DC) and in vitro suspension culture was observed in a case of acute unclassified leukemia (null-AL) and t(4;17). Prior to culture, the blast cells were TdT and Ia positive but did not express any lineage-specific antigenic markers. Furthermore, the immunoglobulin heavy chain and T cell receptor β -chain genes were in germline configuration. Cytogenetically, all metaphases had the unique translocation t(4;17) (q25;q23) prior to and after culture, supporting the leukemic origin of the cells. During both DC culture and suspension culture with and without tetradecanoyl-phorbol-acetate (TPA), a substantial increase in the absolute and relative number of cells expressing both myeloid and T lymphoid antigenic

markers occurred. Double-fluorescence analysis demonstrated the expression of antigenic markers of both lineages on the same population of cells, and electron microscopy revealed the induction of myeloperoxidase after both DC and suspension culture. Immunoglobulin heavy chain and T cell receptor β -chain genes remained in germline configuration after treatment with TPA, when analyzed with JH and CT β probes, respectively. These findings indicate that this case represents a null-AL with dual-lineage capabilities, which has probably arisen from the malignant transformation of a bipotential stem cell of lymphoid and myeloid progeny.

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SEVERAL recent reports have shown that in rare instances acute leukemic blast cells may express morphological, cytochemical, immunologic, or molecular genetic features of both the T lymphoid and one of the myeloid lineages.¹⁻⁶ Rapid conversion from a T lymphoid to a myeloid phenotype during treatment further supported the hypothesis that in some of these cases the leukemia arose from a multipotent stem cell capable of both lymphoid and myeloid differentiation.^{3,5,6}

We report a case of null-AL with the unique karyotype 46,XY,t(4;17) that originally did not exhibit any features of commitment. The cells were capable, however, of expressing markers of the myeloid and T lymphoid lineage after diffusion chamber (DC) and suspension culture without and with 12-O-tetradecanoyl-phorbol-acetate (TPA).

MATERIALS AND METHODS

Patient. On initial examination, a 64-year-old man had a leukocyte count of 400,000/ μ L with 98% blasts. His bone marrow was packed with blast cells. The blasts were negative for myeloperoxidase, α -naphthyl acetate esterase (ANAE), periodic-acid Schiff (PAS), and acid phosphatase. They were TdT positive but negative for lineage-specific surface markers. The diagnosis of null-AL was made. The patient died of cerebral leukostasis 1 week after an initial dose of 2 mg of vincristine intravenously (IV) and 35 mg of prednisolone three times daily orally.

Cells. After informed consent was given by the patient, leukemic blast cells were isolated from the peripheral blood by separation on a Ficoll-Hypaque gradient (1.077 g/mL) enriching the percentage of blast cells to >99%. The cells were cryopreserved in 10% dimethylsulfoxide at -196°C in liquid nitrogen prior to the present studies.

Diffusion chamber technique. The DC technique has previously been described.^{7,8} In brief, DC filled with 7.7×10^5 cells were implanted into CBA/Ca mice that had been given 7.5 Gy whole body X-irradiation. The chambers were harvested by shaking for 30 minutes in 0.5% pronase B (Merck, Darmstadt, FRG) in buffered Hank's solution to liquefy the clot. The resulting single-cell suspension harvested from 8 to 14 chambers at each interval was pooled. Smears were made for differential counts, and the remaining cells were used for immunological analysis. Cells to be used for surface marker analysis after being harvested from DC were further incu-

bated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Conco Laboratories, Wiesbaden, FRG) for 2 hours at 37°C in 5% CO_2 . The incubation in pronase needed for harvesting the chamber cells had no effect on the expression of the antigens examined and evaluated in the present experiments, as tested with positive and negative controls.

Suspension culture. The leukemic cells were cultured in vitro for 3 and 6 days at 1×10^6 cells/mL in RPMI-1640 supplemented with 50 μ mol/L of 2-mercaptoethanol, 10% heat-inactivated FCS, 2 mmol/L of glutamine, and antibiotics at 37°C in a 5% CO_2 humidified atmosphere. TPA (Sigma, St Louis) dissolved in ethanol at 10^{-5} mol/L was stored at -20°C and diluted in culture medium just before use to give a final concentration of 5×10^{-9} mol/L. The final concentration of ethanol in the control cultures was <0.1%. At the end of the incubation period, aliquots were taken for enumeration of viable cells by trypan blue exclusion and for phenotypic analysis.

Phenotypic analysis with antibodies. Phenotypic analysis was performed with the cells settled onto glass slides coated with poly-L-lysine.⁹ Attached cells were fixed for 7 minutes in glutaraldehyde (0.05% in 0.1 mol/L of phosphate buffer, pH 7.4) at 4°C followed by 10 minutes in 0.3 mol/L of glycine buffer. The cells were then tested with monoclonal antibodies against differentiation antigens of the various cell lineages: OKIa1 against HLA-DR¹⁰; the T

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cell markers CD7[T, gp41]WT-1,¹¹ CD1[Thy, gp45, 12]OKT6,¹² CD3[T, gp19-29]OKT3,¹² CD4[T, gp56-62]OKT4,¹² CD8[T, gp32-33]OKT8¹²; the myelomonocytic markers CDw13[M, G, U]MY-7,¹³ CDw14[M, gp55] MY-4,¹³ MY-9,¹⁴ CDw15[G, CHO]B4.3,¹⁵ B13.9,¹⁵ VIM-2,¹⁶ and OKM1¹⁷; the markers CD10 common acute lymphoblastic leukemia antigen (CALLA) [nT-nB, gp100]J5¹⁸ and BA-3¹⁹; the B lineage-associated markers CD19, B4,²⁰ pan B,²¹ CD24 [p45, 55, 65]BA-1,²² CD9[nT-nB, p24]BA-2,²³ and a monoclonal antibody against IgM (P.M. Lansdorp, unpublished observations); the stem cell marker RFB-1, also reactive with pre-T cells²⁴; a megakaryocytic marker C17.28 directed against platelet glycoprotein IIIa²⁵; an erythroid marker VIE-G4 against glycophorin,²⁶ B3/25²⁷ against the transferrin receptor; Tac against the interleukin-2 (IL-2) receptor,²⁸ and the pan-leukocyte marker T29/33.²⁹ The antibodies B4.3, B13.9, anti-IgM, and C17.28 were provided by P.M. Lansdorp, Amsterdam; VIM-2 and VIE-G4 by W. Knapp, Vienna; RFB-1 was provided by M.P. Bodger, Auckland; WT-1 by W. Tax, Nijmegen, and Tac was provided by T.A. Waldmann, Bethesda, MD. OK1a1, OKT3, OKT4, OKT6, OKT8, and OKM1 were purchased from Ortho Pharmaceutical, Raritan, NJ; MY-4, MY-7, MY-9, J-5, and B4 from Coulter, Hialeah, FL; BA-3, BA-1, BA-2, B3/25, and T29/33 from Hybritech, San Diego; and panB from Dakopatt, Copenhagen. The cluster designations (CDs) are those recommended at the First and Second International Workshops on Human Leukocyte Differentiation Antigens.³⁰

Binding of antibodies to target cells was revealed by the immunalkaline phosphatase method³¹ using goat-anti-mouse Ig coupled to alkaline phosphatase (Sigma, 1:40) as second antibody. Isotype-identical nonreactive mouse monoclonal antibodies served as controls. Cytoplasmic μ chains were detected by a modification of the method of Vogler and colleagues³² using a monoclonal antibody against μ chains (P.M. Lansdorp, Amsterdam) and the alkaline phosphatase antialkaline phosphatase (APAAP) technique. Reagents used as sandwich antisera in the APAAP technique consisted of the goat-anti-mouse Ig coupled to alkaline phosphatase (Sigma, 1:20) and the APAAP complex from the mouse (Dakopatts). TdT was assayed by a modification of the method of Bollum.³³ The reagents for the terminal deoxynucleotidyl transferase (TdT) assay were purchased from BRL (Gaithersburg, MD); 200 cells were examined in each test.

Double-fluorescence analysis. Double-fluorescence immunologic phenotyping was done prior to and after DC and suspension culture, using the antibodies VIM-2, WT1, and OKT6. Cells were first labeled with the myeloid antibody VIM-2 and counterstained with TRITC-conjugated, affinity-purified F(ab')₂ fragment goat-anti-mouse IgM (μ chain-specific, dilution 1:20; Zymed Laboratories, Wiesbaden, FRG), followed by incubation with the T lymphoid markers WT1 or OKT6 and counterlabeling with FITC-conjugated, affinity-purified F(ab')₂ fragment goat-anti-mouse IgG (Zymed). The fluorescence of the cells was examined under a Leitz microscope with epifluorescent illumination and equipment for the dual-wavelength method; 200 cells were examined in each test.

Cytogenetic analysis. Cytogenetic analysis was performed before and after DC and suspension culture as already described,³⁴ using GTG-banding.

Immunoglobulin heavy chain and T cell receptor β chain gene rearrangements. DNAs were extracted from mononuclear cells after Ficoll-Hypaque centrifugation by standard techniques. DNA 15 μ g was digested with appropriate restriction enzymes (Boehringer Mannheim), electrophoresed on a 0.7% agarose gel, and blotted and hybridized as described.^{35,36} λ -DNAs were included as mol wt markers. Bgl/II digests were hybridized to a JH probe (Oncor, Gaithersburg, MD), which detects a 5.0-kilobase (kb) heavy chain Ig gene germline fragment. BamHI digests were hybridized to a C τ β probe (Oncor) that detects a 23-kb germline band of the T cell

receptor β chain gene. Probes were handled under P2EK1 containment according to the National Institute of Health Guidelines for research involving recombinant DNA molecules. After hybridization, the filters were washed under stringent conditions and exposed to XAR-5 film (Kodak) using Dupont Lightning-Plus intensifying screens for 12 to 48 hours at -70°C .

Electron microscopy. Ultrastructural analysis for the detection of peroxidase was done on cells that were either unfixed³⁷ or fixed in glutaraldehyde³⁸ prior to incubation in 3'-3'-diaminobenzidine (DAB) and H₂O₂. For controls, either DAB or H₂O₂ was omitted from the incubation mixture. The processing for transmission electron microscopy was carried out as described by Polli and colleagues.³⁹ Sections were viewed unstained according to usual procedures.

In situ hybridization. According to published protocols,⁴⁰ chromosomes were treated with RNase and denatured in 70% formamide/2 $\times$ SSC at 70°C for 2 minutes. Probes were nick-translated using ³H-dCTP and ³H-dTTP (NEN, Boston) to a specific activity of 1.9×10^7 cpm μg^{-1} , denatured for 5 minutes at 70°C in 50% formamide/2 $\times$ SSC/10% dextran sulfate and hybridized for 12 hours at 37°C ; slides were rinsed in 50% formamide/2 $\times$ SSC followed by extensive washings in 2 $\times$ SSC and exposed to Kodak NTB 2 emulsion for 10 days at 4°C , developed, and stained with quinacrine mustard. The probe used was a 1.8-kilobase (kb) EcoRI p53 cDNA clone (p53-H13).⁴¹

RESULTS

Prior to in vitro culture, the leukemic blast cells did not express lineage-specific markers but were positive for OK1a and the stem cell marker RFB-1. The T cell marker WT-1 was positive in <5% and the myeloid marker MY-7 was positive in <15% of the cells. Morphologically, the blast cells were undifferentiated, with occasional to moderate numbers of nucleoli. The cytochemical reactions were negative; ultrastructurally, no myeloperoxidase or platelet peroxidase was detectable (Figs 1a and 2a). The cells were TdT-positive, however. Southern blot analysis of the immunoglobulin and the T cell receptor genes showed no rearrangement within the immunoglobulin μ gene and the T cell receptor β chain sequences covered by the probes used (Fig 3). Cytogenetic analysis revealed a t(4;17)(q25;q23) translocation in all 25 metaphases analyzed (Fig 4). After hybridization of human p53 sequences to 43 metaphases, 153 grains were observed on chromosomes. The distribution of the silver grains was uniform and random on all chromosomes, except for specific signals ($P < .001$) on chromosomes 17 (18 grains v 2.4 grains expected) and 17q+ (21 grains v 3.1 expected); 21% of grains clustered on region 17p13 on both chromosomes.

The cells were cultured using two different types of culture systems. During DC culture, the total number of cells increased substantially, rising from 3.85×10^6 cells/mL ($7.7 \times 10^5/\text{DC}$) to 8.5×10^6 cells/mL ($17.0 \times 10^5/\text{DC}$). After 14 days of DC culture, 17.8% of the cells could be morphologically identified as belonging to the granulopoietic lineage (Table 1). There was a rapid loss of the marker RFB-1, followed by a relative and absolute increase in the number of cells expressing MY-7, VIM-2, B4.3, B13.9, MY-4, and OKM-1 (Table 2). The cells expressing the T-marker WT1 also increased steadily, whereas the number of cells reactive with OK1a and those carrying the transferrin

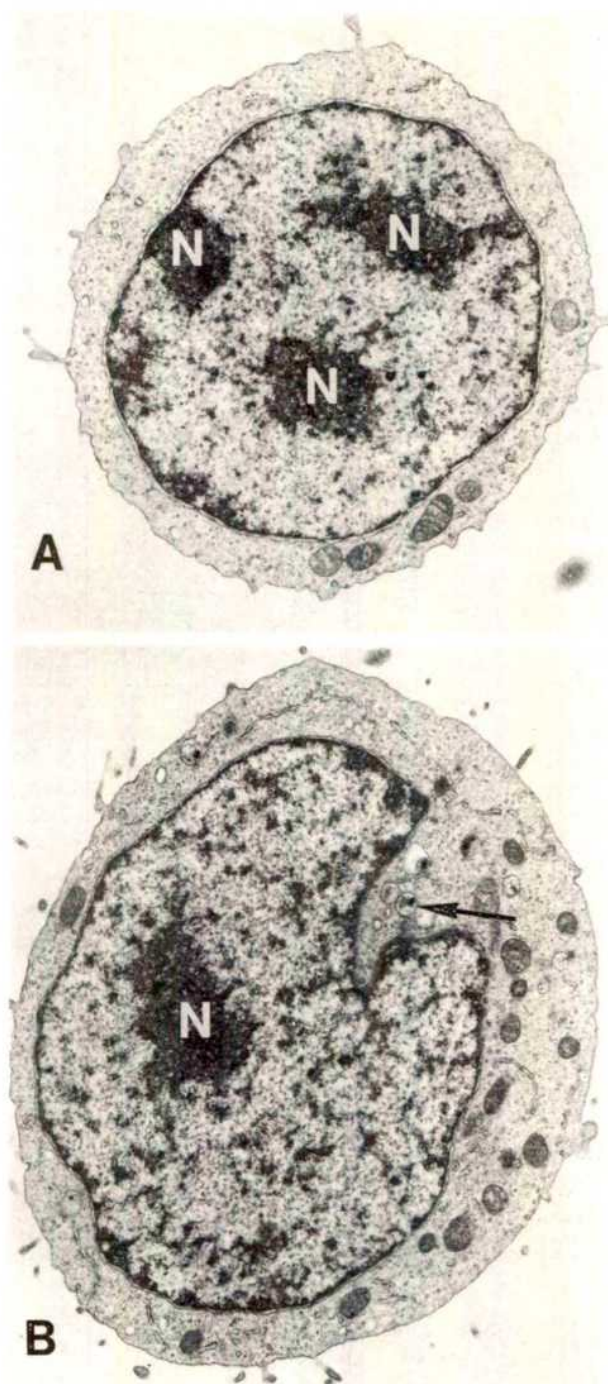


Fig 1. Ultrastructural morphology of the blast prior to (A) and after (B) culture. Prior to cultures the blasts have the morphology of undifferentiated, lymphoblastlike cells with several prominent nucleoli (N) lacking morphological features of myelomonocytic differentiation (lead citrate stain, original magnification $\times 12,000$; current magnification $\times 9,000$). After culturing, the nucleus becomes more indented. The enlarged cytoplasm contains many mitochondria and several vesicles; some contain electron-dense inclusion bodies (arrow in b). No azurophilic granules are visible. (N, nucleolus; lead citrate stain; original magnification $\times 10,000$; current magnification $\times 7,500$).

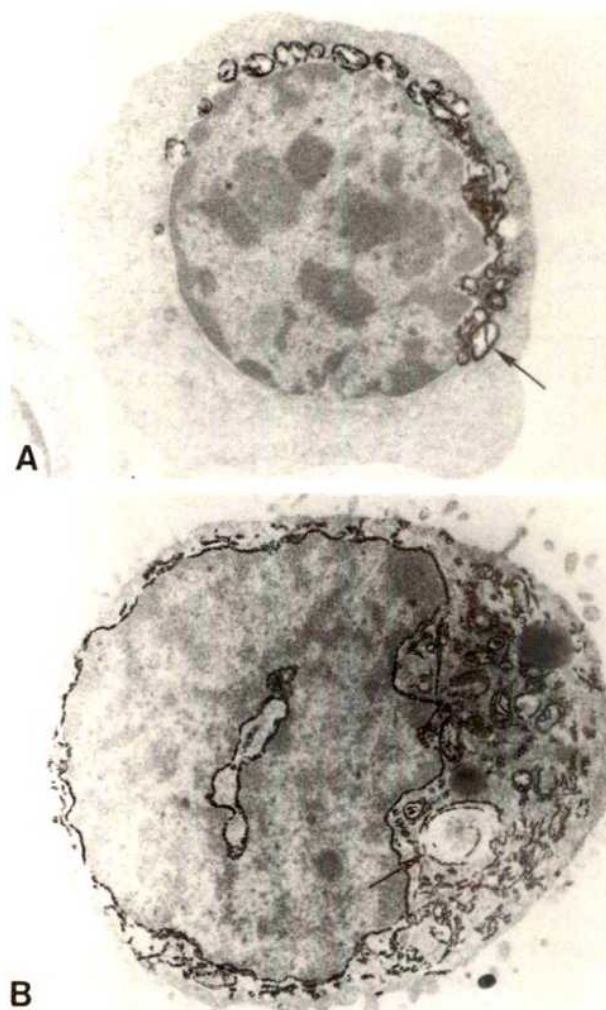


Fig 2. Ultrastructural cytochemistry (platelet peroxidase [PPO] reaction) of the blast cells, unstained sections. (A) Prior to culture the blast cells are undifferentiated, only a nonspecific peroxidase reaction can be seen in the mitochondria (arrow) due to oxidative phosphorylation enzymes (original magnification $\times 12,600$; current magnification $\times 9,450$). (B) After exposure to tetradecanoyl-phorbol-acetate (TPA) for 3 days, the blast cells developed the characteristic pattern of peroxidase activity of myeloblasts: reactivity is seen in the nuclear envelope, in the endoplasmic reticulum, and in the Golgi cisternae (arrow). The two large dark staining spots represent fat droplets in the cytoplasm (original magnification $\times 13,000$; current magnification $\times 9,750$).

receptor decreased both in absolute and relative numbers during the culture period.

During suspension culture, the cells retained their blast-like morphology; however, $\sim 25\%$ of the cells developed focal acid phosphatase positivity under either condition, whereas no myeloperoxidase was detected at the light microscope level. The number of cells staining with MY-7 (Table 3) increased substantially within 24 hours (data not shown). Another myeloid marker, VIM-2, also developed. When the cells were exposed to TPA, the pan-T marker WT1 and the IL-2 receptor (Tac) were newly expressed, followed by early (OKT6) and late (OKT3, OKT4) T cell markers, as well as BA-2. A proportion of cells (25%) also became focally

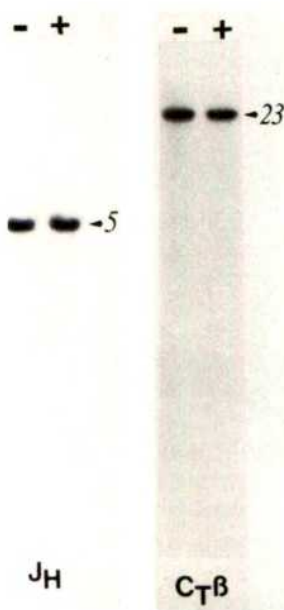


Fig 3. Southern blot analyses of immunoglobulin heavy chain and T cell receptor β chain rearrangements before (–) and after (+) suspension culture in presence of tetradecanoyl-phorbol-acetate (TPA). DNAs from leukemic cells were analyzed for IgH and T β rearrangements after digestion with *Bgl*II and *Bam*HI, respectively, using the probe indicated at the bottom of each blot. The numbers at the side of the lanes are the size of fragments in kilobases.

positive when stained cytochemically for acid phosphatase. Again no myeloperoxidase was observed at the light microscope level, however. The phenotype of the cells then was MY-7+, VIM-2+, BA-2+, OKT6+, OKT3+, OKT4+, B3/25+, OKIa+ (Table 3). No B lineage-specific markers were expressed under any culture conditions. Simultaneous staining using double-immunofluorescence revealed that after culture with TPA both myeloid and T lymphoid markers were expressed on the same population of cells (Table 4).

Because the cells were Tac-antigen positive after exposure

Table 1. Morphological Analysis of Cells Prior to and After DC Culture

Duration of DC Culture (days)	Nucleated Cells/DC ($\times 10^{-6}$)	Blast Cells (%)	Immature Granulopoietic Cells (M ₁₋₄) [*] (%)	Mature Granulopoietic Cells (M ₅₋₈) [†] (%)
0	7.7	97.4	—	—
1	5.1	99.6	—	—
7	12.8	99.8	—	—
14	17.0	67.4	12.7	5.1

DC, diffusion chamber.

^{*}M₁₋₄: myeloblasts, promyelocytes, myelocytes.

[†]M₅₋₈: metamyelocytes, juveniles, band forms, segmented granulocytes.

to TPA, the cells were then further incubated with either IL-2 or concanavalin A, alone, or in combination for another 4 days. No further increase in cell number was observed after either culture, however (data not shown).

Ultrastructural analysis revealed that although the cells were negative prior to culture, >80% expressed peroxidase activity in the nuclear envelope, the endoplasmic reticulum, and the Golgi apparatus after DC culture and after suspension culture with TPA (Fig 2b). When the cells were analyzed cytogenetically, only pseudodiploid metaphases with the karyotype 46,XY,t(4;17) (q25;q23) were found after any of the various cultures. The immunoglobulin heavy chain and the T cell receptor β chain gene regions remained in the germline configuration when probed with the respective probes (Fig 3), after exposure of the cells for 4 days to TPA in suspension culture.

DISCUSSION

Several characteristics of the cells prior to and after culture indicate that in this case the malignant transforma-

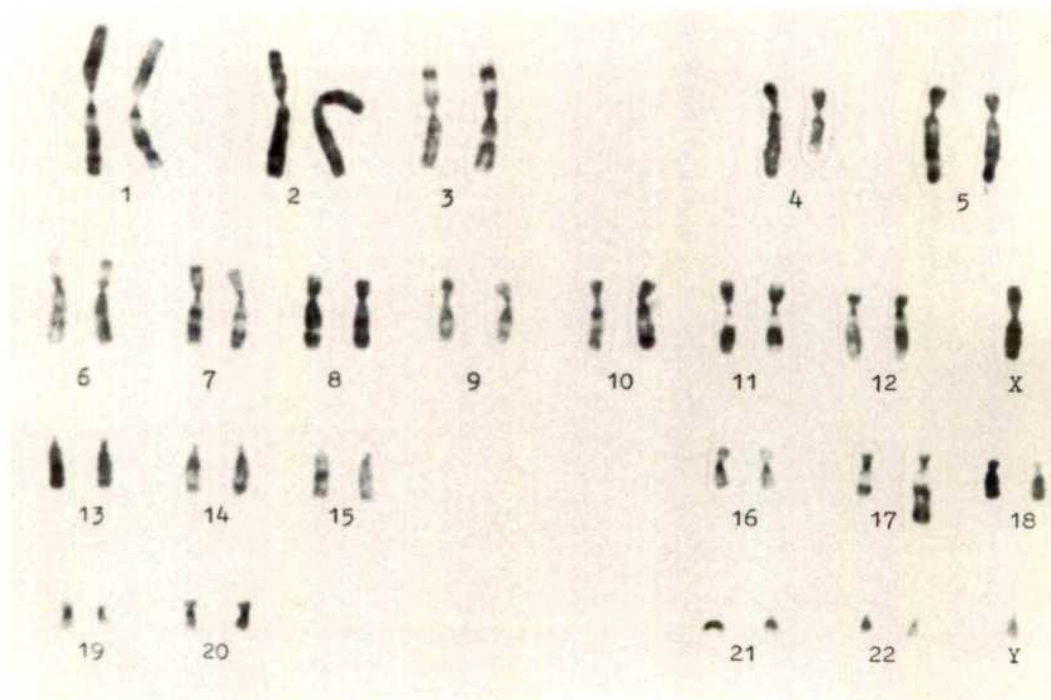


Fig 4. Karyotype of unstimulated blood cells showing t(4;17) (q25;q23).

Table 2. Immunologic Analysis of Cells Prior to and During DC Culture

Duration of DC Culture (Days)	Phenotypic Markers (%)													
	My-AG							Mo-Ag		T-Ag				
	RFB-1	BA-2	MY-7	VIM-2	MY-9	B4.3	B13.9	My-4	OKM-1	WT-1	B-Ag	Tac	B3/25	OKIa
0	45	36	15	2	0	0	0	0	0	4	0	0	25	68
1	0	0	82	0	0	0	0	4	4	10	0	4	47	100
7	0	57	41	70	0	33	3	43	50	36	0	12	32	38
14	21	35	98	79	38	46	22	37	96	29	22	5	4	46

DC, diffusion chamber.

My-Ag: myelopoiesis-associated antigens; Mo-Ag, monocytic antigens; T-Ag, T cell-associated antigens; B-Ag: B cell-associated antigens (B4, pan B). BA-1, VIE-G4, C17.28, cIgM, SIgM, J5, BA-3, OKT3, and OKT6 were negative.

Table 3. Immunological Characteristics of the Blast Cells From Null-AL Prior to and After Suspension Culture

Days in Culture	Inducing Agent	Cells/mL ($\times 10^{-5}$)	Reactivity With Monoclonal Antibodies (%)													
			RFB-1	BA-2	MY-7	VIM-2	B4.3	MY-9	MY-4	OKM-1	J5	WT-1	OKT6	OKT3	OKT4	Tac
0	—	10.0	45	36	15	2	0	0	0	5	0	4	0	0	0	0
3	—	15.0	23	23	79	72	7	0	0	6	0	35	4	2	7	19
6	—	38.0	7	43	61	22	0	0	10	14	0	25	12	7	12	6
3	TPA, 5 nmol/L	10.9	48	55	91	65	0	12	0	8	0	53	2	0	0	48
6	TPA, 5 nmol/L	8.8	20	79	80	40	ND	7	14	42	0	20	45	34	51	0

TPA, tetradecanoyl-phorbol-acetate. ND, not done.

tion could have taken place in a cell capable of myeloid and T lymphoid differentiation: the initial expression of RFB-1, a marker found on hemopoietic progenitor cells and very immature T cells but not on B lineage cells,²⁴ the induction of both T lymphoid and myeloid markers, the induction of focal expression of acid phosphatase and, finally, the induction of myeloperoxidase. The occurrence of a malignant translocation at this level has been previously postulated by the findings of rapid switches from T-ALL to acute myeloid leukemia.^{3,5,6,42}

The present case differs from previous cases of multilineage differentiation⁴³ by the absence of differentiation markers on the cells prior to culture. An unusual differentiation capacity was revealed by both the in vivo and in vitro culture systems, however. During DC culture, which allows maturation along the myeloid^{7,8} and lymphoid lineages,⁴⁴ the cells not only expressed several markers of the myeloid as well as the T lymphoid lineages, but also partially differentiated to morphologically recognizable granulocytic cells. Differentiation toward the myeloid lineage was supported by transmission electron microscopy, demonstrating the presence of

myeloperoxidase in the blast cells after DC culture as well as after exposure to TPA in suspension culture.⁴⁵

Although the induction of T cell markers, including the thymic marker OKT6, is very unusual and to our knowledge has never been found in a case of null-AL before,^{46,47} there has been a case of acute myeloid leukemia that expressed WT-1 before and OKT6 after in vitro culture.⁴⁸ This was interpreted as lineage infidelity with aberrant expression of lymphoid surface antigens on myeloid cells; however, the apparent similarity to our case suggests a capacity for bilineage differentiation in both cases.

Evidence that both myeloid and T lymphoid markers were coexpressed on a significant proportion of the blast cells was provided by expression of the myeloid markers MY-7 and OKM-1 on nearly all and of WT-1 on about one-third of the cells after DC culture. Further evidence is given by the results of the double-labeling analysis after suspension culture, which demonstrated the coexpression of both the myeloid marker VIM-2 and the T lymphoid markers WT-1 and OKT6 on the same blast cell population.

Because WT1 can occasionally be found on leukemic cells of the myeloid lineage,⁴⁹ it was of importance that further T-cell markers OKT6, OKT3 and OKT4¹² were inducible by TPA in suspension culture. That the immunoglobulin heavy chain and T cell receptor β chain gene regions remained in the germline configuration after exposure to TPA and that T cell marker appeared on the surface of treated cells indicates that no clonal D-J joining events occurred in these cells after induction of differentiation with TPA.

The proliferation of normal cells from the peripheral blood is largely excluded by cytogenetic analysis before and after culture, by the coexpression of myeloid and T cell markers on the same cells, and by the expression of OKT6.⁵⁰ Although cytogenetic analysis provides the karyotype of only a small number of cultured cells, the remote possibility that cells other than those with t(4;17) could have contributed to the unique marker expression can be largely ruled out. This

Table 4. Expression of Myeloid (VIM-2, MY-7), T Lymphoid (WT-1, OKT6), and IL-2 Receptor (Tac) After Exposure to 5 nmol/L of TPA for 72 hours as Determined by Double Fluorescence

Monoclonal Antibodies	Positive Cells (%)
VIM-2	65
MY-7	32
VIM-2 + MY-7	65
WT-1	14
VIM-2 + WT-1	50
OKT6	0
VIM-2 + OKT6	34
Tac	1
VIM-2 + Tac	37

IL-2, interleukin-2; TPA, tetradecanoyl-phorbol-acetate.

would imply a rapid increase in cell number, which should have been detected by cytogenetic analysis.

The observed chromosomal translocation allows a provocative speculation about the role of chromosomes 4 and 17 in the present and other cases of bilineage differentiation. A translocation with a breakpoint on chromosome 4, ie, t(4;11), has been described in acute leukemia of unclassified phenotype,^{51,52} in acute lymphoblastic leukemia,⁵³ and in acute leukemias with both myeloid and lymphoid features.^{54,55} In addition, the breakpoints on chromosomes 4 and 17 have both been described in a case of null-AL and variant translocation t(4;11;17).⁵⁶ Thus, the possibility that these chromosome regions are of importance, perhaps related to an

oncogene, is intriguing. Although involvement of the gene for p53 could be ruled out in concordance with a recently published report,⁵⁷ the significance of the gene for erb A located on the long arm of chromosome 17⁵⁸ or the gene coding for IL-2 on chromosome 4⁵⁹ remains an area for further investigations.

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Platelet Surface-Bound IgG in Patients With Immune and Nonimmune Thrombocytopenia

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We quantitated the amount of platelet surface-bound IgG using an ^{125}I monoclonal anti-IgG assay in 149 patients with thrombocytopenia and 260 normal donors. The normal subjects had 122 ± 5 molecules of IgG/platelet (mean $\pm$ SE). Fifty-five patients with nonimmune thrombocytopenia had 338 ± 37 molecules of IgG/platelet, whereas 67 patients with immune thrombocytopenia studied at the time of their initial evaluation had $4,120 \pm 494$ molecules of IgG/platelet. An analysis of the distribution of values in these two groups indicated that 90% of the patients with immune thrombocytopenia had >800 molecules of IgG/platelet, whereas only 7% of patients with nonimmune thrombocytopenia exceeded this amount. The immune thrombocytopenic patients included 39 idiopathic, 14 secondary, and 14 drug-induced disorders, and they did not significantly differ in their distribution of values for platelet IgG. The nonimmune thrombocytopenic patients included 12 cases with a platelet destructive mechanism; their

platelet-bound IgG was similar to that of the other nonimmune patients. Twenty-seven patients with treatment-resistant immune thrombocytopenia were also studied; they had $2,100 \pm 670$ molecules of IgG/platelet. Their values were significantly greater than those of the nonimmune thrombocytopenic patients and not significantly different from those of immune thrombocytopenic group. Their distribution of values was much broader, however, with 33% of patients having <800 molecules of IgG/platelet, suggesting possible alternate mechanisms in their thrombocytopenia. Thus, patients with immune thrombocytopenia have a high frequency of elevated IgG on the platelet surface which reflects the pathophysiology of this disorder. Quantitation of platelet-bound IgG provides a useful laboratory tool in the differential between immune and nonimmune thrombocytopenia.

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IMMUNE thrombocytopenic purpura (ITP) in adults is a syndrome of shortened platelet survival with reticuloendothelial sequestration. This syndrome of accelerated platelet destruction appears to be mediated primarily by IgG antibody directed toward ill-defined platelet surface antigens.^{1,2} In the decade since Dixon and co-workers³ first documented elevated levels of platelet-bound IgG (PBIgG) in patients with ITP, a variety of assay techniques for measuring platelet IgG has been reported.⁴⁻¹³ These different assays use various methodologies and report elevated values for IgG associated with platelets in ITP. Elevated values have also been noted in thrombocytopenia presumed to be due to nonimmune mechanisms, however, resulting in the interpretation that such values are a nonspecific finding unrelated to the pathophysiology of immune platelet destruction.¹⁴⁻²⁰ Recent studies have clarified the limitations imposed by conventional competitive binding assays, using calibration by soluble IgG standards^{21,22} and the potential value of a direct binding assay using stoichiometric relationships. We have previously characterized and described such an assay using monoclonal ^{125}I anti-human IgG to quantify platelet surface-bound IgG.^{23,24} By means of this quantitative technique the amount of surface IgG displayed on the

platelet surface in a large group of thrombocytopenic patients was analyzed.

MATERIALS AND METHODS

Assay for platelet surface-bound IgG. The preparation of monoclonal ^{125}I anti-IgG reagent as well as the standard assay conditions are reported in detail elsewhere.²⁴ This antibody was originally generated by J. Donald Capra and was purchased from Bethesda Research Laboratory (MD), but is no longer available from this source. More recently, the hybridoma producing this antibody is available through the American Type Culture Bank (ATCC-HB-43), and purified antibody is commercially available. (Southern Biotechnology Associates, Birmingham, AL, catalogue no. 9040). In brief, venous blood was collected in plastic syringes and anticoagulated with acid citrate dextrose A. This method allows shipment of whole blood on wet ice with minimal effect on platelet IgG levels.²⁵ The whole blood was centrifuged at 200 g for 20 minutes, and the platelet-rich plasma was collected. The pH was adjusted to 6.5 with acid citrate dextrose A and then centrifuged at 1,750 g for 20 minutes. The platelet button was washed three times in platelet-suspension buffer (Dulbecco's phosphate-buffered isotonic saline containing 1 g/dL of bovine serum albumin (BSA) and 4 mmol/L of EDTA, pH 6.7). Ten million washed platelets were incubated with 300 ng of ^{125}I -labeled mouse monoclonal anti-IgG in a total volume of 300 μL for 20 minutes at room temperature. Triplicate aliquots of 75 μL (2.5×10^6 platelets) were transferred to Percoll gradient tubes containing 250 μL of isotonic 30% Percoll in saline, centrifuged at 7,500 g for 5 minutes, and the tube tip containing the platelet button was transected with a hemostat and razor blade into γ counting tubes. The number of ^{125}I monoclonal antibody molecules bound per platelet was calculated on the basis of Avogadro's number, the antibody's specific activity, and mol wt. We previously showed that with these assay conditions the stoichiometry of the binding reaction approaches unity and that the "nonspecific" binding of the monoclonal anti-IgG is $<5\%$.²⁴ Thus, the number of molecules of platelet-bound ^{125}I monoclonal antibody is equal to the number of molecules of platelet surface-bound IgG.²⁴

Patient characteristics. One hundred forty-nine thrombocytopenic patients with at least three platelet counts $<100,000/\mu\text{L}$ and 260 normal subjects comprised the study group. Sixty-seven of the patients were referred for evaluation at the time of initial diagnosis of immune thrombocytopenic purpura based on conventional clinical

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Table 1. Platelet Counts and Surface-Bound IgG in 12 Patients With Nonimmune Destructive Thrombocytopenia

Patient	Diagnosis*	Platelet Count (μ L)	Molecules IgG/Platelet
1. J.H.	TTP	5,000	723
2. R.M.	TTP	5,000	158
3. T.H.	TTP	8,000	1,612
4. D.E.	TTP	9,000	136
5. M.G.	TTP	16,000	489
6. D.S.	TTP	18,000	149
7. I.G.	TTP	57,000	423
8. D.D.	TTP	63,000	211
9. R.M.	MHA	12,000	274
10. J.O.	DIC	29,000	341
11. J.K.	DIC	33,000	228
12. C.C.	DIC	73,000	239

*TTP, thrombotic thrombocytopenic purpura; MHA, microangiopathic hemolytic anemia; DIC, disseminated intravascular coagulation.

criteria (adult onset thrombocytopenia with normal to increased megakaryocytes in the bone marrow and no other explanation for thrombocytopenia found). Of this group, 39 were classified as idiopathic, 14 were secondary (7 systemic lupus erythematosus, 3 rheumatoid arthritis, and single cases of lymphoma, infectious mononucleosis, Evans syndrome, and AIDS), and 14 were drug-induced (11 quinidine and single cases with reserpine, mezlocillin, and chlorpropamide). These patients were studied prior to therapy or after no more than 48 hours of initiation of corticosteroids. Fifty-five patients had thrombocytopenia that was considered non-immune in origin, including nonimmune destructive thrombocytopenia in 12 patients (Table 1), aplastic anemia in 13, acute leukemia in 11, hypersplenism in 6, multiple myeloma in 3, metastatic cancer in 3, hereditary thrombocytopenia in 2, sepsis in 2, renal disease in 2, and paroxysmal nocturnal hemoglobinuria in 1 patient.

A third group of thrombocytopenic patients was classified as treatment-resistant ITP. These 27 patients had the criteria for idiopathic thrombocytopenic purpura, were treated extensively elsewhere, and were referred for evaluation because of persistent thrombocytopenia. All these patients had received weeks to months of corticosteroids, many had been splenectomized, and some had received immunosuppressive therapy.

Statistical analysis. The distributions of platelet-bound IgG values in various groups of thrombocytopenic patients and normal subjects were compared. The significance of the difference between any two of these distributions was determined by the Wilcoxon two-sample test. Sensitivity and specificity were calculated as measurements of the diagnostic ability of platelet-bound IgG to discriminate immune thrombocytopenia from nonimmune thrombocytopenia. The correlation coefficient and the significance of the correlation between platelet counts and platelet-bound IgG values were calculated using Spearman's method.

RESULTS

Our observations of platelet-bound IgG in various patients and normal groups is summarized in Table 2. In a group of 260 normal volunteers studied for a 2-year period, the mean amount of platelet-bound IgG was 122 ± 5 (mean $\pm$ SE) with a median of 92 molecules of IgG per platelet. The highest value recorded was 400 molecules of IgG per platelet. The 55 patients with nonimmune thrombocytopenia had higher mean (338 ± 37) and median (260 molecules per platelet) amounts of platelet-bound IgG that were significantly ($P < .001$) above that seen in normal controls by the Wilcoxon two-sample test (Table 3). There was a modest negative correlation of platelet count and platelet-bound IgG with a Spearman coefficient of correlation of -0.285 ($P = .037$).

As shown in Table 2, the patient groups of immune thrombocytopenia at the time of initial evaluation as well as the group of treatment-resistant immune thrombocytopenia had dramatically greater amounts of platelet-bound IgG than did either the normal donors or nonimmune thrombocytopenic patients. The patients with idiopathic, secondary, and drug-induced immune thrombocytopenia did not differ significantly from each other in regard to platelet-bound IgG values (Table 3). As a group or individually, however, they had significantly greater amounts of platelet-bound IgG than did patients with nonimmune thrombocytopenia or normal donors (Table 3).

Figure 1 examines the proportion of patients having various amounts of platelet-bound IgG. To differentiate between patients with immune thrombocytopenia v nonimmune, a cutoff value of 800 molecules of IgG per platelet provided the best sensitivity and specificity values. Sixty of 67 patients with immune thrombocytopenia at the time of initial evaluation had >800 molecules of IgG per platelet (sensitivity 90%), whereas 51 of 55 patients with nonimmune thrombocytopenia had <800 molecules of IgG per platelet (specificity 93%). Values of <400 molecules of IgG per platelet adequately describe the population of normal donors. Table 2 provides a breakdown of all patient groups according to these cutoff values. Thus, 90% of patients with immune thrombocytopenia had platelet-bound IgG >800 molecules of IgG per platelet whereas 7% of nonimmune thrombocytopenic patients had values above this cutoff.

The group of patients with nonimmune thrombocytopenia included 12 patients with thrombocytopenia due to premature destruction of platelets (nonimmune) and presumably increased platelet production similar to the platelet kinetics

Table 2. Platelet-Bound IgG in Thrombocytopenic Patients and Normal Donors

Group	Molecules of IgG per Platelet				
	Mean $\pm$ SE	Median	<400 (%)	400-800 (%)	>800 (%)
Immune thrombocytopenia (ITP) (n = 67)	4,120 $\pm$ 494	2,597	4	6	90
Idiopathic (n = 39)	4,420 $\pm$ 706	2,847	8	5	87
Secondary (n = 14)	4,896 $\pm$ 096	2,597	0	7	93
Drug-induced (n = 14)	2,508 $\pm$ 643	1,498	0	7	93
Treatment-resistant ITP (n = 27)	2,100 $\pm$ 670	1,387	15	18	67
Nonimmune thrombocytopenia (n = 55)	338 $\pm$ 37	260	69	24	7
Normal donors (n = 260)	122 $\pm$ 5	92	99.6	0.4	0

Table 3. Significance Tests of Differences in the Distributions of Surface-Bound IgG Between Groups

Comparisons	P
Immune thrombocytopenia (ITP)	
Idiopathic v secondary	.579
Idiopathic v drug-induced	.254
Secondary v drug-induced	.103
Immune thrombocytopenia v normal donors	<.0001
Immune v nonimmune thrombocytopenia	<.0001
Immune thrombocytopenia v treatment-resistant ITP	.090
Treatment-resistant ITP v normal donors	<.0001
Treatment-resistant ITP v nonimmune thrombocytopenia	<.0001
Nonimmune thrombocytopenia v normal donors	<.0001

Wilcoxon two-sample test was used for significance testing.

that exist in immune thrombocytopenia. As listed in Table 1, eight of these patients had thrombotic thrombocytopenic purpura. Their distribution of platelet-bound IgG was similar to that of the other nonimmune thrombocytopenic patients, with 67% in the normal range (<400), 25% between 400 to 800, and 8% (one patient) >800 molecules of IgG per platelet. This patient had received large volumes of plasma prior to referral to our institution, which may have influenced the result at the time of our study.

The group of patients with treatment-resistant immune thrombocytopenia had all been extensively treated prior to referral to our institution. This is obviously a highly selected subgroup of immune thrombocytopenia. As a group, their platelet-bound IgG was significantly greater than that of nonimmune thrombocytopenic patients or normal donors (Table 3). Their amount of platelet surface-bound IgG was almost significantly lower than that of the group of patients with immune thrombocytopenia, however ($P = .09$). In

addition, the values for platelet-bound IgG were more widely distributed than those of the patients studied at the time of initial diagnosis (Table 2 and Fig 2). A third of these patients had <800 molecules of IgG per platelet, whereas some of the patients had large amounts of IgG on their platelet surfaces (Fig 2).

Table 4 lists the characteristics of the patients whose platelet-bound IgG did not correlate with the clinical diagnosis of immune v nonimmune etiology. We were unable to discern any clinical parameters that characterized the patients with immune thrombocytopenia who had low platelet antibody values as compared with their counterparts with elevated values, including appropriate response to therapy. Three of the patients with nonimmune thrombocytopenia had values minimally >800 molecules of IgG per platelet cutoff, and the fourth patient had TTP with substantial plasma infusions prior to study.

Finally, as illustrated in Fig 3, we found no correlation between platelet count and platelet-bound IgG values in the 67 patients with immune thrombocytopenia ($r = -.063$, $P = .612$).

DISCUSSION

The controversy regarding the amount and pathophysiologic role of immunoglobulin associated with platelets from normal and immune thrombocytopenic patients is gradually being clarified. Using a variety of assay methods, most earlier studies suggested that normal platelets had large amounts of platelet-associated IgG (5,000 to 80,000 molecules per platelet) and even higher levels in immune thrombocytopenia.³⁻²⁰ These very large and seemingly nonphysiologic amounts of platelet-associated IgG have led some investigators to question the role of the IgG so measured in the pathophysiology of immune destruction.¹⁴⁻¹⁷ Some stud-

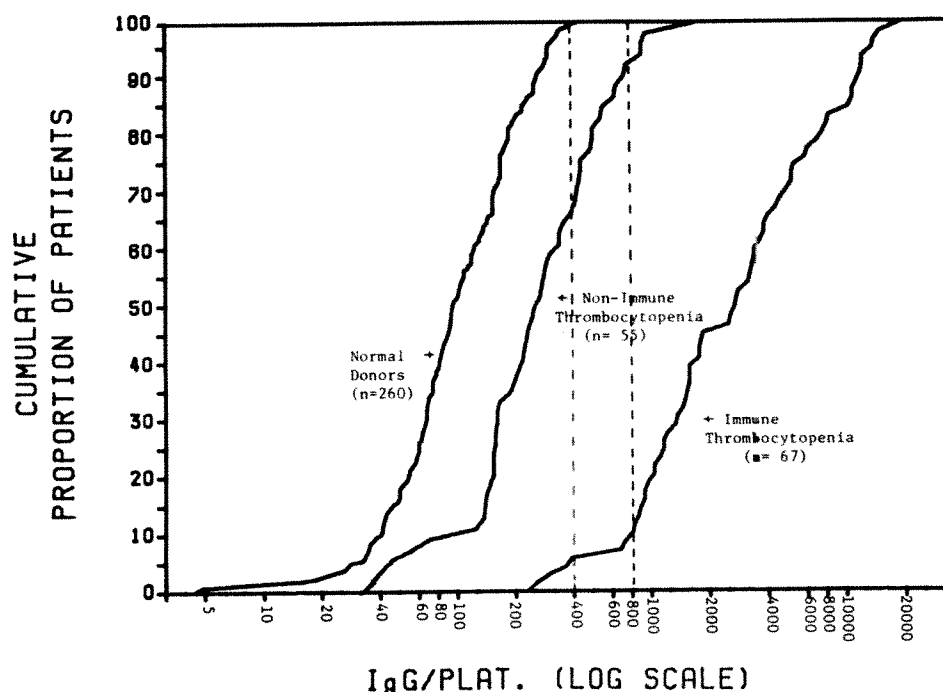


Fig 1. Proportion of patients with various amounts of platelet surface-bound IgG in normal and thrombocytopenic groups. The amounts of platelet surface-bound IgG are expressed as molecules of IgG per platelet (log scale).

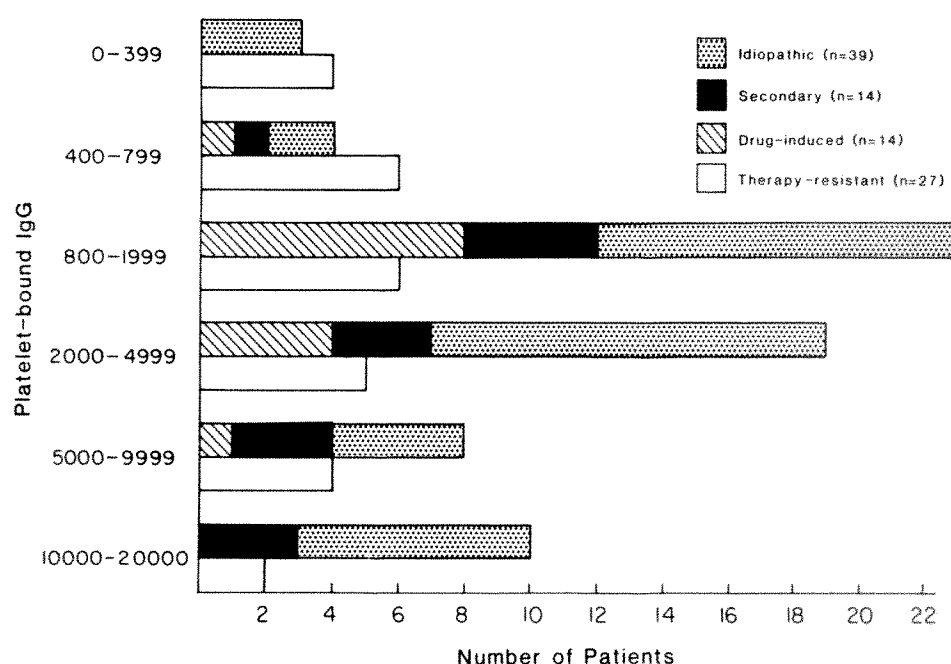


Fig 2. Platelet surface-bound IgG in 94 patients with immune thrombocytopenia. Patients evaluated at the time of initial diagnosis, upper bar; patients referred because of treatment-resistant immune thrombocytopenia, lower clear bar at each level of platelet-bound IgG.

ies have explored the result of differences in assay and calculation technique on estimates of platelet IgG. Rosse and colleagues²¹ and Blumberg and colleagues²² recently suggested that anti-IgG has 40 to 80 times the affinity for surface-displayed IgG as compared with soluble IgG, thus accounting for the very high values of platelet IgG when competitive binding assays calibrated with soluble IgG standards are used. Another tenable hypothesis is that certain assays purposely or inadvertently^{9,12,13,26} cause platelet lysis or release of canalicular entrapped plasma, resulting in measurement of both surface and "intracellular" IgG. In this regard, we used the monoclonal anti-IgG assay to demonstrate that the normal platelet surface displays only a few hundred IgG molecules but that platelet lysates have large amounts (20,000 to 80,000 molecules of IgG per platelet), comparable to results of prior reports.²⁴ To provide truer

estimates of platelet-bound IgG, certain assay techniques directly measure anti-IgG binding and use stoichiometric calculations to quantify platelet surface-bound IgG. These reports include the monoclonal anti-IgG assay,^{21,23,24,27} staphylococcal protein A assay,²⁸ and an assay using a polyclonal anti-IgG.¹¹ All these studies demonstrate that normal platelets have relatively little surface IgG, and amounts ranging from 500 to 13,000 molecules of IgG per platelet are present in immune thrombocytopenia. These amounts of platelet surface-bound IgG are similar to the values seen in the well-studied pathophysiology of warm immune hemolytic anemia.^{29,30}

Of these techniques, we believe that the monoclonal anti-IgG assay has several advantages. First, the monoclonal anti-IgG reacts equally with all four subclasses of IgG,²⁴ whereas staphylococcal protein A reacts poorly or not at all with IgG₃ and the polyclonal reagent has not been characterized and may vary with each antiserum produced. Second, the monoclonal anti-IgG has a very high binding affinity,^{24,27}

Table 4. Divergent Clinical Diagnosis and Platelet-Bound IgG Values

Patient	Diagnosis	IgG/Platelet	Platelet Count
Immune thrombocytopenia			
R.S.	Idiopathic	251	50,000
E.E.	Idiopathic	350	12,000
M.H.	Idiopathic	380	38,000
R.M.	Idiopathic	671	16,000
J.W.	Idiopathic	702	89,000
E.H.	Drug, chlorpropamide	766	22,000
D.H.	Infectious mononucleosis	790	10,000
Nonimmune thrombocytopenia			
J.C.	Aplastic anemia	852	20,000
J.H.	Non-Hodgkin's lymphoma	861	26,000
R.W.	Breast cancer, marrow metastasis	893	22,000
T.H.	TTP	1612	8,000

TTP, thrombotic thrombocytopenic purpura.

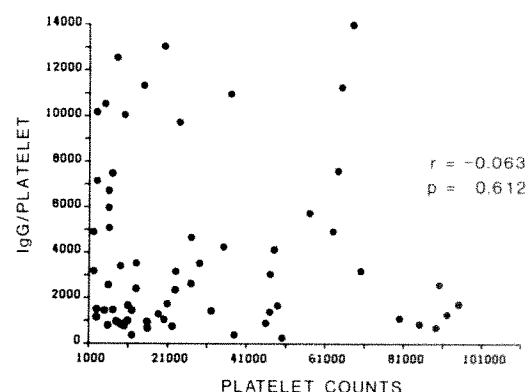


Fig 3. Correlation of platelet count and amount of platelet surface-bound IgG in 67 patients with immune thrombocytopenia at the time of initial diagnosis.

allowing saturation kinetics to be reached at low protein concentrations (1 ng/ μ L) under our assay conditions, resulting in little or no nonspecific anti-IgG association with the platelet surface.²⁴ Finally, the use of a single Percoll gradient centrifugation to separate platelet-bound from free anti-IgG precludes loss of platelets and disassociation of anti-IgG from IgG during repeated washing steps. The major drawback with this technique has been the relative unavailability of the monoclonal anti-IgG reagent. This is no longer a problem because the hybridoma cell line is available from the American Type Culture Bank (ATCC-HB-43) and from a commercial source of the purified antibody (Southern Biotechnology Associates).

In this study, we examined the amount of platelet surface-bound IgG in patients with thrombocytopenia due to immune and nonimmune etiologies. We showed that 90% of patients with immune thrombocytopenic purpura have increased amounts of surface-bound IgG (>800 molecules of IgG per platelet) at the time of initial diagnosis with a range of 87% to 93% for the various subgroups (Table 2). In contrast, patients with nonimmune thrombocytopenia infrequently had elevations of this degree (7%) indicating that quantitation of platelet surface-bound IgG can be a valuable adjunct in the evaluation of thrombocytopenic patients. Prior reports have indicated that patients with TTP have elevated platelet associated IgG.^{14,31} Our experience in eight patients failed to confirm this observation: only one patient had an elevated value, which was suspect because of prior exposure of the patient to large amounts of pooled plasma before being referred for study. Indeed, the patients with nonimmune thrombocytopenia associated with enhanced platelet destruction (and presumably enhanced platelet production) had low amounts of platelet-bound IgG, similar to those of other nonimmune thrombocytopenic disorders.

The group of patients with treatment-resistant immune

thrombocytopenia differed from their counterparts at initial diagnosis in that a larger proportion of them (33%) had values of platelet surface IgG comparable to those of normal donors or nonimmune thrombocytopenia patients. This may reflect the role of other pathophysiologic processes, including IgM, complement, or nonimmune mechanisms.

Two caveats to interpretation of platelet-bound IgG values in thrombocytopenic patients should be noted. Patients with immune thrombocytopenia who have been on therapy for >48 hours, usually with rising platelet counts, may have normal platelet-bound IgG values. Conversely, patients with acute leukemia who are severely ill and are receiving multiple antibiotics and platelet transfusions often have elevated values, the mechanisms of which are unknown but may be multifactorial. We have excluded both of these groups from our analysis and do not recommend interpretation of platelet-bound IgG values in these circumstances.

Finally, studies carried out in our laboratory³² using normal platelets sensitized in vitro with an IgG anti-PIA₁ antibody indicated that human monocytes have a threshold of ~1,000 molecules of IgG per platelet for recognition and binding of IgG-coated platelets through their Fc receptors. This is certainly compatible with the degree of sensitization observed in this study of platelets sensitized in vivo (ITP) resulting in presumed macrophage binding in splenic sinusoids.

Thus, this study indicates that patients with immune thrombocytopenia have a high frequency of elevated amounts of platelet surface-bound IgG as compared with patients with nonimmune thrombocytopenia. Quantitation of surface-bound IgG represents a clinically useful tool for delineating an immune etiology related to IgG on the platelet surface. The finding of elevated platelet-bound IgG by this monoclonal anti-IgG technique certainly appears to be of pathophysiologic relevance.

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Measurement of Free, One-Chain Tissue-Type Plasminogen Activator in Human Plasma With an Enzyme-Linked Immunosorbent Assay Based on an Active Site-Specific Murine Monoclonal Antibody

By P. Holvoet, J. Boes, and D. Collen

An enzyme-linked immunosorbent assay for free tissue-type plasminogen activator (t-PA) in human blood was developed based on a murine monoclonal antibody directed against the active site of t-PA. The lower limit of sensitivity of the assay applied to plasma is 2 ng/mL for one-chain t-PA but only 100 ng/mL for two-chain t-PA. Free t-PA in plasma taken at rest was found in 6 of 21 healthy subjects (4.5 ± 0.8 ng/mL, mean $\pm$ SD) and increased to 14 ± 7.0 ng/mL after venous occlusion in 18 of these individuals. A linear correlation between total t-PA and free t-PA was observed with $r = 0.92$ ($n = 18$) and a slope of 1.08,

indicating that t-PA released from the vessel wall circulates in the blood as the one-chain form. In 16 of 18 patients with deep vein thrombosis, the increase of total t-PA antigen after venous occlusion was comparable to that observed in controls, but the free t-PA was significantly lower or undetectable. The present assay for free t-PA may be useful for the investigation of the release and inhibition of t-PA under physiological, pharmacological, or pathological conditions in humans.

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TISSUE-TYPE plasminogen activator (t-PA) constitutes a physiologically important pathway for the activation of the fibrinolytic system in blood.¹ Venous thrombosis is frequently associated with a reduced increase of plasminogen activator activity in the blood, following standard stimuli (eg, venous occlusion).^{2,3} This impaired fibrinolytic response may be due to the rapid inhibition of released t-PA by a potent fast-acting inhibitor of plasminogen activator (PA-inhibitor).⁴

In earlier studies, the measurement of the plasminogen activator activity in plasma relied on the measurement of the fibrinolytic activity in euglobulin fractions.⁵ Such assays lack specificity, however, because they are dependent on many factors, such as the concentration of plasminogen activators, plasminogen, fibrinogen, and inhibitors. More recently, functional assays more specific for t-PA were developed.^{6,7} In these relatively complicated assays, the presence of fibrin or fibrinogen/fibrin fragments is required, and the results depend on the adequate removal of inhibitors.

Immunoassays specific for t-PA have also been developed.⁸⁻¹⁴ Although these assays are very sensitive (allowing detection of t-PA in the pmolar range), they do not discriminate between functionally active and complexed forms of t-PA. Indeed, the observed increase in total t-PA antigen following venous occlusion in patients with venous thrombosis was not consistently associated with an increase in t-PA activity.^{11,14} In such patients, increased levels of the fast-acting inhibitor of plasminogen activator have been measured.¹⁵⁻¹⁷

Immunoassays that could discriminate between free (ac-

tive) and complexed (inactivated) t-PA antigen might be useful for a more detailed investigation of the release and inhibition of t-PA in blood, under physiological, pharmacological, and pathological conditions. In the present study, we developed an enzyme-linked immunosorbent assay (ELISA) specific for free t-PA in blood. This assay was applied to the study of the release and inhibition of t-PA following venous occlusion in plasma samples of healthy subjects and patients with deep vein thrombosis.

MATERIALS AND METHODS

Purified proteins. One-chain and two-chain t-PAs were purified from the culture fluid of human melanoma cells.¹⁸ The activity was expressed in international units by comparison of the fibrinolytic activity on plasminogen-enriched bovine fibrin films⁵ with that of the 1st International Reference Preparation of t-PA (83/517).¹⁹ The protein concentration of t-PA was determined by amino acid analysis.¹⁸ In this way, a specific activity of ~500,000 IU/mg was obtained for purified t-PA.

Cell hybridization and screening of hybridomas. Balb/c mice were immunized by subcutaneous and intraperitoneal injections of purified t-PA. P3X63-Ag.8-6.5.3. myeloma cells²⁰ were fused with spleen cells from immunized mice.²¹ After selection in HAT medium (Dulbecco's modified Eagle medium [DMEM] containing hypoxanthine, aminopterin, and thymidine),²² the supernatants were screened for specific antibody production in micro-ELISA²³ using microtiter plates coated with t-PA. The bound immunoglobulins were quantitated with horseradish peroxidase-conjugated rabbit anti-mouse IgG. Positive clones were subcloned by limiting dilution. The effect of monoclonal antibodies on the fibrinolytic activity of t-PA (final concentration 15 nmol/L) was determined by incubation of t-PA with a 40-fold molar excess of antibody for 1 hour at 37 °C followed by application of the 10- μ L aliquots of the mixture to plasminogen-containing fibrin plates.⁵ Monoclonal antibodies directed against the active site of t-PA were identified by their differential reactivity toward active t-PA or t-PA in which the active center was blocked by either diisopropylfluorophosphate (DFP) (final concentration 1 mmol/L) or D-Ile-Pro-Arg-CH₂Cl²⁴ (final concentration 10^{-5}).

The amidolytic activity of t-PA (final concentration 25 nmol/L) before and after preincubation with a 40-fold molar excess of monoclonal antibodies was measured spectrophotometrically at 405 nm using the synthetic substrate D-Ile-Pro-Arg-p-nitroanilide (S-2288) (final concentration 0.3 mmol/L).²⁵

Preparation of polyclonal antisera. Rabbit antisera against t-PA were produced by four biweekly subcutaneous injections of 50

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μg of purified t-PA once in complete Freud's adjuvant and then in incomplete adjuvant. The IgG fractions were purified by affinity chromatography on protein A-Sepharose.²⁶

Blood collection and treatment of plasma samples. Venous blood samples were collected from 21 control subjects in 0.1 vol of 0.11 mol/L of citrate or in citrate containing MA-2G6 (final concentration 20 $\mu\text{g}/\text{mL}$). The samples were centrifuged at 3,000 g and 4 °C and immediately assayed or stored at -20 °C until use. Venous occlusion was performed by inflating a cuff around the upper arm, between systolic and diastolic blood pressure, for 10 minutes.

Blood samples were also obtained from 18 patients (10 men and 8 women, age 56 ± 17 years) with phlebographically confirmed deep vein thrombosis, which could not be attributed to malignancies, liver disease, infections, or a postoperative state. In three patients, the venous thrombosis was complicated by pulmonary embolism. Thirteen patients were studied between the second and fourth day after the initial diagnosis, while on heparin anticoagulation. The other five patients were studied 3 weeks after the initial diagnosis, while on oral anticoagulant therapy. Pooled fresh human plasma was depleted in t-PA by immunoadsorption on immobilized anti-t-PA monoclonal antibody (MA-62E8).¹⁴

ELISA specific for free, one-chain t-PA. The IgG fraction purified from polyclonal rabbit antiserum against t-PA was diluted in 0.04 mol/L of phosphate buffer pH 7.4, containing 0.14 mol/L NaCl, 10 g of polyethylene glycol 6,000, and 60 g saccharose per liter (coating solution).⁹ Samples (200 μL) of a 4 $\mu\text{g}/\text{mL}$ solution were incubated for 48 hours at 4 °C in the wells of polyvinylchloride microtiter plates (Costar, Cambridge, MA). The fluid was then decanted, the wells were treated for 2 hours at room temperature with 200 μL of coating solution containing 10 g/L bovine serum albumin (BSA), with 200 μL of coating solution without BSA and finally with a solution containing 10 g of mannitol and 20 g of saccharose per liter. Immediately before use, the microtiter plates were washed once with 0.04 mol/L of phosphate buffer pH 7.4, containing 0.14 mol/L of NaCl (PBS). Plasma samples collected in the presence of MA-2G6 (final concentration 20 $\mu\text{g}/\text{mL}$) were diluted in PBS-Tween-BSA, and dilutions of samples to be assayed (180 μL) were incubated for 16 hours at 4 °C in a moist chamber. The wells were then emptied and washed (three cycles in a Skatron microwash, Analis, Namen, Belgium) with PBS containing 0.002% Tween 80 (PBS-Tween). Then, to quantitate the bound t-PA-antibody complexes, 180 μL of a horseradish peroxidase-conjugated rabbit anti-mouse IgG solution diluted 1:2,000 in PBS-Tween containing 2% of normal rabbit serum was added and the mixture was incubated for 1 hour at room temperature. After repeated washing (three cycles) of the plates, the peroxidase reaction was performed by addition of 180- μL aliquots of a 0.1 mol/L of citrate-0.2 mol/L of sodium PBS pH 5.0, containing 200 $\mu\text{g}/\text{mL}$ of o-phenylenediamine and 0.003% hydrogen peroxide. After 30 minutes at room temperature, the reaction was arrested with 50 μL of 4 mol/L of sulphuric acid. The absorbance was measured at 492 nm with a multiskan spectrophotometer (Titertek, Flow Laboratories, Irvine, Scotland). Standard curves for the ELISA were constructed using t-PA-depleted plasma to which purified human one-chain t-PA (final concentration 50 ng/mL) was added. To this mixture, MA-2G6 (final concentration 20 $\mu\text{g}/\text{mL}$) was added. After preincubation for 2 hours at 4 °C, the t-PA-antibody complexes were quantitated as described above.

Inhibition of t-PA by endothelial cell-derived PA-inhibitor. Human endothelial cells were prepared from umbilical cord veins, and confluent cultures were stimulated with endotoxin as described.²⁷ Growth medium and conditioned medium of these cells were gel-filtered on a $2.5 \times 80\text{-cm}$ column of Sephadex G-200 after addition of one-chain t-PA (final concentration 0.2 $\mu\text{g}/\text{mL}$). The column was equilibrated with 0.015 mol/L of phosphate, 0.3 mol/L

of NaCl, 0.05 mol/L of EDTA, pH 7.5, containing 10 KIU/mL of aprotinin and 0.01% Tween 80. The flow rate was 6 mL/h and 4-mL fractions were collected. Relative mol wt markers were human IgGs, mol wt 140,000; phosphorylase b, mol wt 98,000; and human albumin, mol wt 66,000.

ELISA for total t-PA. A two-site ELISA reacting equally well with free and complexed forms of one-chain and two-chain t-PA was performed as described earlier.¹⁴

Other techniques. Polyacrylamide gel electrophoresis (PAGE)²⁸ was performed as described elsewhere. Amino acid analysis was performed on a Beckman 119 CL amino acid analyzer after hydrolysis in 6 mol/L of HCl in vacuo at 110 °C for 20 hours.

Reagents. Urokinase (Winkase) was a gift from Dr G. Murano, Bureau of Biologics, Bethesda, MD. Aprotinin (Trasylol) from Bayer (Leverkusen, FRG), thrombin (Topostasine) from Roche (Basel, Switzerland), and bovine plasminogen-containing fibrinogen from Organon (Oss, The Netherlands) were used. CNBr-activated Sepharose and diethylaminoethanol (DEAE) Affi Gel Blue were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The synthetic inhibitor D-Ile-Pro-Arg-CH₂Cl was obtained by custom synthesis at UCB (Brussels). The chromogenic substrate D-Ile-Pro-Arg-p-nitroanilide was obtained from KabiVitrum (Amsterdam). Horseradish peroxidase-conjugated rabbit anti-mouse IgG was obtained from Nordic (Tilburg, The Netherlands), and o-phenylenediamine was obtained from Fluka (Buchs, Switzerland). The P3X63-Ag.8-6.5.3. myeloma cells were a gift from Dr O. Schönher (Organon).

RESULTS

Identification of anti-t-PA monoclonal antibodies. Supernatants of hybridomas resulting from fusions of P3X63-Ag.8-6.5.3. myeloma cells with spleen cells from Balb/c mice, immunized with purified t-PA, were screened for specific antibody production in a one-site micro-ELISA using t-PA-coated plates. Nine fusions yielded 36 hybridomas producing monoclonal antibodies reactive with different epitopes in the t-PA molecule as determined by the method of Stähli.²⁹ One monoclonal antibody (MA-2G6) quenched both the fibrinolytic and amidolytic activity of t-PA when added in a 40-fold molar excess. Because this antibody bound to free t-PA but not to active site-blocked t-PA, it was used to develop the assay for free t-PA. This antibody is characterized in more detail elsewhere.³⁰

ELISA specific for free t-PA. The wells of the polyvinyl microtiter plates were coated with varying concentrations (between 0.06 and 32 $\mu\text{g}/\text{mL}$) of the IgG fraction of the t-PA-specific polyclonal antiserum. Maximal binding of 80 ng of t-PA-MA-2G6 complexes occurred at concentrations >2 $\mu\text{g}/\text{mL}$ (not shown). An IgG concentration of 4 $\mu\text{g}/\text{mL}$ was subsequently used in the assay. The influence of the concentration of the horseradish peroxidase-conjugated rabbit anti-mouse IgG was studied with dilutions between 1:1,000 and 1:12,000. Maximal response was obtained in dilution of $\leq 1:4,000$. A conjugate dilution of 1:4,000 (final IgG concentration 0.8 $\mu\text{g}/\text{mL}$) was selected for the assay (not shown).

One-chain t-PA was added to citrated plasma to a concentration of 100 ng/mL, and increasing concentrations of MA-2G6 dissolved in PBS-Tween-BSA (between 2 and 32 μg IgG/mL of plasma) were added. The mixtures were incubated for 2 hours at 4 °C, diluted in the same buffer, and

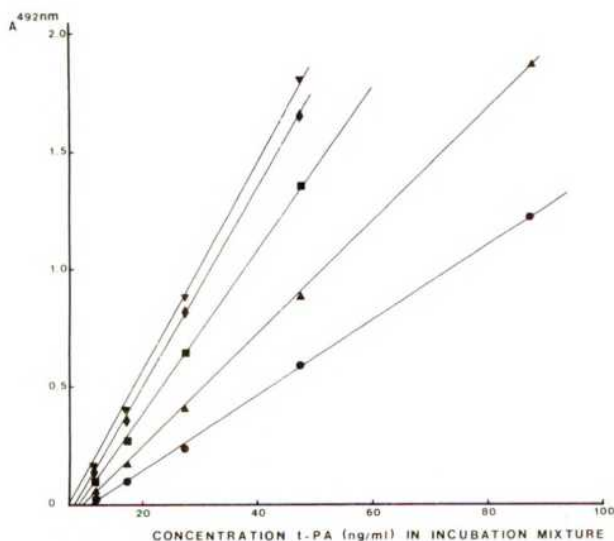


Fig 1. Influence of the concentration of murine monoclonal antibody MA-2G6 on the sensitivity of the assay. One-chain tissue-type plasminogen activator (t-PA) (final concentration 100 ng/mL) was preincubated in the presence of increasing concentrations of MA-2G6: 2 µg/mL (●); 4 µg/mL (▲); 8 µg/mL (■); 16 µg/mL (◆); and 32 µg/mL (▼). The samples were diluted in phosphate-buffered saline, Tween, and bovine serum albumin (PBS-Tween-BSA), and the t-PA-MA-2G6 complexes were quantified with horseradish peroxidase-conjugated rabbit antimouse IgG.

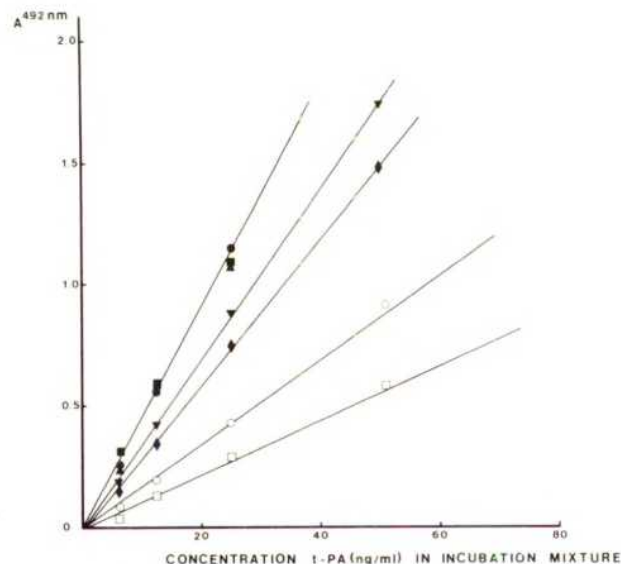


Fig 2. Influence of plasma matrix effects on the detection of tissue-type plasminogen activator (t-PA). One-chain t-PA (final concentration 50 ng/mL) was added to pooled normal human plasma dilutions: 1:1 (□); 1:2 (○); 1:4 (◆); 1:8 (▼); 1:16 (▲); 1:32 (■); and buffer (●). Murine monoclonal antibody MA-2G6 (final concentration 20 µg/mL), was then added, and dilutions of the mixtures in phosphate-buffered saline, Tween, and bovine serum albumin (PBS-Tween-BSA) (final t-PA concentration in the samples between 2.5 and 0.1 ng/mL) were used for quantitation of t-PA-antibody complexes with horseradish peroxidase-conjugated rabbit anti-mouse IgG.

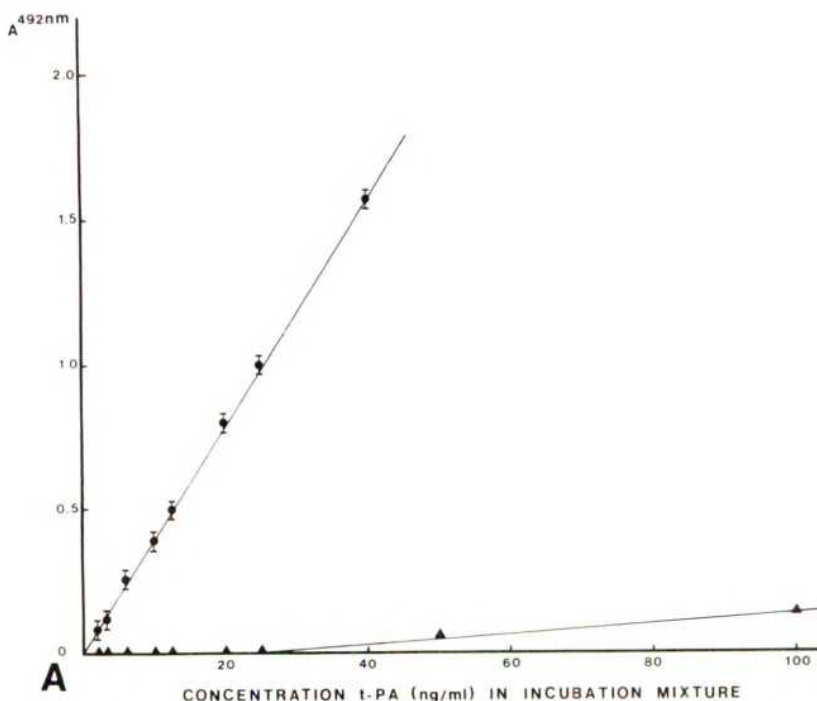


Fig 3. (A) Reactivity of one-chain tissue-type plasminogen activator (t-PA) and two-chain t-PA in the assay for free t-PA. Calibration curves for the quantitation of one-chain t-PA (●) in human plasma were obtained by addition to t-PA depleted of t-PA (final concentration 50 ng/mL) and of murine monoclonal antibody MA-2G6 (final concentration 20 µg/mL). The samples were diluted in phosphate-buffered saline, Tween, and bovine serum albumin (PBS-Tween-BSA) (final concentration between 2.5 and 0.08 ng/mL). Poor cross-reactivity of two-chain t-PA (▲) was confirmed by addition of two-chain t-PA (final concentration between 5 and 0.08 ng/mL). (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis: 5 µg of unreduced two-chain t-PA (lane 1), unreduced one-chain t-PA (lane 3), reduced two-chain t-PA (lane 4), and reduced one-chain (lane 6) were applied to 12% slab gels. Electrophoresis was performed overnight at room temperature and 60 V. The proteins were visualized with Coomassie blue. Unreduced and reduced standards with mol wt indicated on the right were applied in lanes 2 and 5.

the t-PA levels were then measured as described above. Figure 1 illustrates the influence of the concentration of monoclonal antibody on the sensitivity of the assay. An antibody concentration of 20 $\mu\text{g/mL}$ was further used in the ELISA.

Standard curves for the measurement of free t-PA in plasma were obtained by dilution of one-chain t-PA (final concentration between 50 and 2 ng/mL) in normal human plasma, in PBS-Tween-BSA, or in mixtures of both. Figure 2 indicates that at low dilution plasma interfered with the assay, whereas this effect was completely eliminated by 16-fold dilution. Therefore, plasma samples were diluted at least 20-fold for the assay of free t-PA. Using the assay conditions described above, between 2 and 40 ng of one-chain t-PA per milliliter of plasma could be adequately measured after 1:20 dilution (Fig 3). Thus, the sensitivity of the assay is 2 ng/mL in undiluted human plasma. Figure 3 further illustrates a large difference in sensitivity of the ELISA for one-chain as compared with two-chain t-PA. Concentrations of 100 ng/mL of two-chain t-PA in the incubation mixture had a reactivity comparable to that of 2 ng of one-chain t-PA.

The reproducibility of the assay was assessed using plasma samples containing 10, 5, and 2.5 ng of one-chain t-PA per milliliter, respectively. The within-assay coefficients of variation were $\sim 3.5\%$ ($n = 20$). The between-assay coefficients of variation ($n = 20$) were 3.0%, 6.5%, and 12.5% for plasma samples containing 10, 5, and 2.5 ng of one-chain t-PA per milliliter, respectively.

The specificity of the assay for free one-chain t-PA was established in the following way. One-chain t-PA, in which the active site was blocked with DFP or with D-Ile-Pro-Arg-CH₂Cl, was not detected in the assay at concentrations of 100 ng/mL. Following gel filtration of unconditioned growth medium used for endothelial cell culture to which 0.2 $\mu\text{g/mL}$ of one-chain t-PA was added, only one peak of immunoreactive material, eluting with an apparent mol wt of 70,000, was detected in both the ELISA for total t-PA and the ELISA based on MA-2G6 (Fig 4A). Following gel filtration of conditioned medium of human endothelial cells stimulated with endotoxin, after addition of 0.2 $\mu\text{g/mL}$ of one-chain t-PA, two peaks of immunoreactive material, corresponding to the elution position of mol wt 110,000 and 70,000, were detected in the ELISA specific for total t-PA. In the ELISA based on MA-2G6, only the material in the mol wt 70,000 position was quantitatively measured (Fig 4B).

In plasma samples of healthy individuals ($n = 21$), taken at rest, the concentration of total t-PA was 4.5 ± 1.3 ng/mL (mean $\pm$ SD) (range 2.1 to 6.9 ng/mL). Detectable levels of free t-PA were found in plasma of only 6 of these 21 healthy subjects (4.5 ± 0.8 ng/mL for free t-PA as compared with 4.9 ± 1.1 ng/mL for total t-PA). After venous occlusion ($n = 21$) measurable plasma concentrations of free t-PA were found in 18 subjects (14 ± 7 ng/mL range 4 to 32 ng/mL). The correlation coefficient (r) between total t-PA and free t-PA determined by linear regression was .92 ($n = 18$) (Fig 5). The slope of the regression line was 1.08, indicating that most of the t-PA antigen following venous occlusion occurred as free one-chain t-PA.

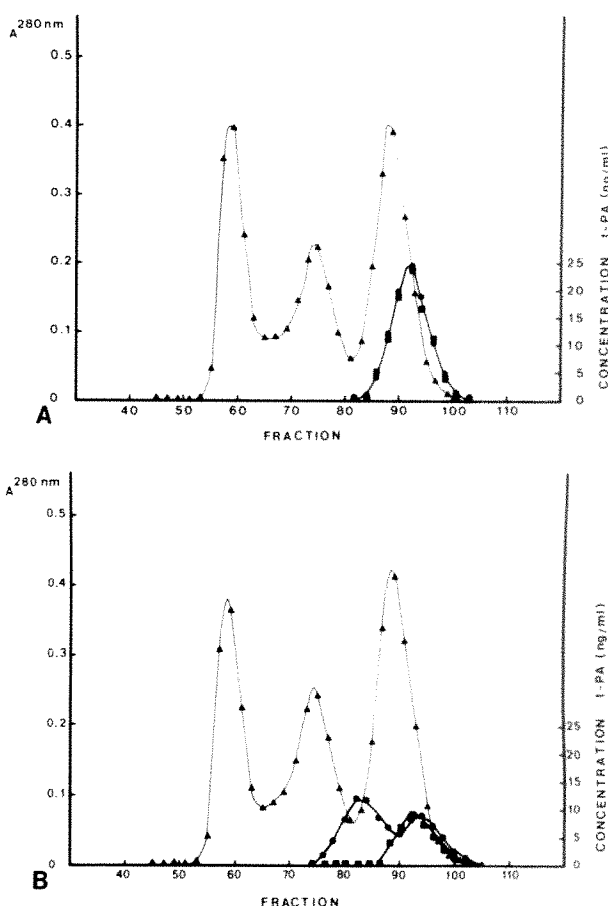


Fig 4. Gel filtration of mixtures of 1.0 μg of tissue-type plasminogen activator (t-PA) and 5 mL of unconditioned (A) or 5 mL of endothelial cell-conditioned medium after stimulation with endotoxin (B). Gel filtration was performed on a 2.5×80 -cm column of Sepharose G-200 equilibrated with 0.01 mol/L of phosphate, 0.3 mol/L of NaCl, 0.005 mol/L of EDTA, pH 7.5, containing 10 KIU/mL of aprotinin and 0.01% Tween 80; 3-mL fractions were collected. Absorbance at 280 nm (▲); total t-PA antigen (ng/mL) (●); free t-PA antigen (ng/mL) (■) measured after addition of murine monoclonal antibody MA-2G6 to the column fractions.

In plasma samples obtained from 18 patients with phlebographically confirmed deep vein thrombosis, the total t-PA antigen concentration was 9.1 ± 2.6 ng/mL (range 5.9 to 12 ng/mL). No free t-PA was detected in any of these samples. After venous occlusion, the total t-PA concentration increased in 16 out of 18 patients to 25 ± 11 ng/mL (range 10 to 51 ng/mL). Free t-PA was detectable in 7 of the 18 patients and increased to 9.1 ± 5.1 ng/mL (range 5.2 to 20 ng/mL) and remained < 2 ng/mL in the 11 other samples (Fig 5).

Following gel filtration of a pool of these 11 samples as described above, only one peak of immunoreactive material corresponding to the elution position of mol wt 110,000 was detected in the ELISA specific for total t-PA.

DISCUSSION

Of 36 t-PA-specific murine monoclonal antibodies, one (MA-2G6), directed against an epitope that overlaps the

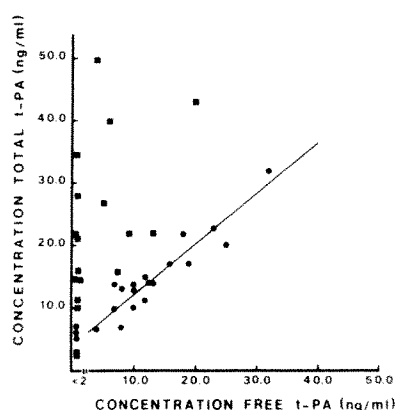


Fig 5. Correlation between total tissue-type plasminogen activator (t-PA) antigen levels and free t-PA antigen levels in plasma after venous occlusion. Control subjects ($n = 21$) (●); patients with deep vein thrombosis ($n = 18$) (■).

active site of t-PA, quenched both the fibrinolytic and amidolytic activity of t-PA. Because this antibody does not react with t-PA, in which the active site is blocked with either the synthetic inhibitor D-Ile-Pro-Arg-CH₂Cl or with the fast-acting PA-inhibitor of endothelial cell origin, MA-2G6 proved useful for the specific measurement of free t-PA with an ELISA. t-PA in concentrations between 2 and 40 ng/mL could be measured adequately after 20-fold dilution of the sample, with between-assay variation coefficients ranging from 3.0 to 12.0%. Conversion of one-chain t-PA into

two-chain t-PA by cleavage of the Arg 275-Ile 276 peptide bond resulted in a 50-fold reduced reactivity of t-PA with MA-2G6.

The ELISA was further used to investigate the release and inhibition of one-chain t-PA in both healthy subjects and patients with phlebographically confirmed deep vein thrombosis. Baseline free t-PA levels could only be measured in 6 of 21 healthy subjects. Venous occlusion, however, resulted in a measurable increase of free t-PA in 18 of the 21 control subjects. A linear correlation between free t-PA and total t-PA was observed, with a slope of the regression line of 1.08. This indicates that t-PA is released from the vessel wall in its one-chain form and that at least in healthy subjects it is not rapidly converted to the two-chain form or quickly neutralized. In a pilot study, the ELISA was used to investigate the release and inhibition of t-PA after venous occlusion in patients with deep vein thrombosis ($n = 18$). Although a normal increase of total t-PA was observed in 16 of these patients, the fraction that was measurable as free t-PA was significantly lower than in control subjects and was undetectable in 11 patients. This observation is compatible with the interpretation that rapid inhibition of the released t-PA occurs in blood and is in agreement with previous results.⁴

We conclude that the present ELISA is specific for free, one-chain t-PA and is sensitive enough for the measurement of free t-PA in plasma. It may be a valuable tool for the investigation of the dynamics of t-PA release and inhibition under physiological, pharmacological, and pathological circumstances. It may also be useful to monitor thrombolytic therapy with one-chain t-PA.

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Megakaryocytopoiesis: Incorporation of Tritiated Thymidine by Small Acetylcholinesterase-Positive Cells in Murine Bone Marrow During Antibody-Induced Thrombocytopenia

By Karen M. Young and Leon Weiss

The ability of small acetylcholinesterase-positive (SACHE) cells to incorporate tritiated thymidine (^3H -TdR) was studied in the bone marrow of mice made acutely thrombocytopenic by injection of guinea pig antmouse platelet serum (APS) and in control mice injected with normal guinea pig serum (NS). ^3H -TdR was administered in vivo, and bone marrow was collected at various time points thereafter. The initial labeling index (LI) for both groups was about 30%. After four hours, the LI increased and reached peak values at 48 hours, but was lower in thrombocytopenic animals. The lower peak LI in APS-treated animals may be the result of both a faster rate of influx of unlabeled cells into the acetylcholinesterase (AChE) positive cell compart-

ment and efflux of more heavily labeled, and probably polyploid, SACHE cells into the compartment of recognizable megakaryocytes. In both groups the increasing LI occurred concomitantly with a decreasing mean grain count. This may indicate cellular division by some fraction of SACHE cells. Heterogeneity in nuclear morphology was also demonstrated, and the frequency of individual morphologies was altered in APS-treated animals. In addition, cells that incorporated ^3H -TdR were larger than those that did not. This group of small cells appears to represent a pivotal point in megakaryocytopoiesis in which the switch from mitosis to endomitosis is occurring.

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IN THE PATHWAY of megakaryocyte differentiation, a group of cells intermediate between the colony forming unit-megakaryocyte (CFU-M) and morphologically recognizable megakaryocytes has recently been detected. In certain species, including the mouse, these cells contain acetylcholinesterase, a cytochemical marker highly selective for megakaryocytes.^{1,2} A proportionate increase in the number of small acetylcholinesterase-positive (SACHE) cells among all acetylcholinesterase-positive cells in bone marrow occurs in response to antibody-induced thrombocytopenia²⁻⁴ and after administration of a thrombopoiesis-stimulating factor,³ and suppression of SACHE cells follows transfusion-induced thrombocytosis.⁵ The early response of these cells indicates that the first detectable response to changes in platelet mass may occur at this level.

SACHE cells appear to play a pivotal role in megakaryocytopoiesis with regard to cellular proliferation/endomitosis and feedback control by platelets, making critical an elucidation of their role in the dynamics of megakaryocytopoiesis. Few recognizable megakaryocytes initially incorporate tritiated thymidine (^3H -TdR), but by 72 hours, greater than 90% of these cells are labeled,^{6,7} even when reutilization of ^3H -TdR is minimized.⁸ Since recognizable megakaryocytes do not divide,^{9,10} we hypothesized that SACHE cells, precursors to recognizable megakaryocytes, would have an initially high labeling index (LI) and that their maturation would result in the subsequent rise in the LI of recognizable megakaryocytes. In addition, we hypothesized that the kinetics would be different in thrombocytopenic animals, since thrombocytopenia has been shown to result in a more rapid

rise in the labeling index of recognizable megakaryocytes^{11,12} and in an increase in the fraction of CFU-M in cycle.^{13,14}

Our studies on the incorporation of ^3H -TdR by SACHE cells indicate a heterogeneity with respect to cell cycle status and suggest that thrombocytopenia results in a more rapid rate of influx of cells from a precursor compartment and/or efflux of SACHE cells into the compartment of recognizable megakaryocytes. In addition we provide information on the morphology of SACHE cells that incorporate ^3H -TdR.

MATERIALS AND METHODS

Mice. BDF₁ mice (Jackson Laboratories, Bar Harbor, ME), 2 to 4 months old, were used in these studies. The animals were maintained under conventional conditions.

Antiplatelet serum. Antiplatelet serum (APS) was the generous gift of Dr Shirley Ebbe (Lawrence Berkeley Laboratory, University of California, Berkeley) and was prepared in the following manner: mouse platelets mixed with 0.15 mol/L sodium chloride (isotonic saline) and equal volumes of complete Freund's adjuvant were injected into guinea pigs. The first injection was given in the foot pad and subcutaneously (SC), and three subsequent injections were given SC only. The injections were administered over a period of one month. One week following the last injection, serum was collected from the guinea pigs and heat-inactivated at 56 °C for 30 minutes. The serum was adsorbed three times with equal volumes of saline-washed mouse red cells and stored at -20 °C in aliquots of 1 mL until use. Thawed aliquots were diluted to 3 mL with isotonic saline, and each of 18 experimental mice received 0.1 mL intraperitoneally (IP). Eighteen control animals each received 0.1 mL of normal guinea pig serum (NS) IP. NS was heated to 56 °C for 30 minutes and diluted to 3 mL with isotonic saline prior to administration.

Tritiated thymidine. Tritiated thymidine (^3H -TdR) was obtained from New England Nuclear (Boston) as a sterile aqueous solution and had a specific activity of 2.0 Ci/mmol. The solution was diluted with sterile isotonic saline and injected IP at a dose of 1.1 $\mu\text{Ci/g}$ body weight four hours after injection with either APS or NS. This time point was chosen because thrombopoiesis-stimulating factor levels have been reported to be high four hours after APS administration.¹⁵

Blood counts. Blood for counts was collected from the retro-orbital sinus directly into potassium-EDTA tubes (Sarstedt, Princeton, NJ). Platelet counts were performed on a calibrated hemocytometer at a dilution of 1:100 with 1% ammonium oxalate. Red blood cell (RBC) and white blood cell (WBC) counts were performed with a Coulter electronic particle counter, Model ZBI.

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Pretreatment blood counts were performed on 26 mice. Counts were repeated four hours after injection with either APS or NS. Platelet counts in APS-treated animals had to be less than 5% of control and pretreatment values in order for the animal to be included in the experiment. This degree of thrombocytopenia appears necessary to effect quantitative changes in SACHE cells.² Exchange transfusions with platelet-poor plasma have been ineffective in producing this degree of thrombocytopenia.^{16,17} No change in RBC or WBC counts occurred in either group of mice. Platelet counts were also done on APS and NS-treated mice at the time of sacrifice.

Bone marrow samples. Animals were killed by cervical dislocation at 30 minutes and at 4, 12, 24, 48, and 72 hours after administration of ^3H -TdR. Bone marrow smears were made from split femora on acid-alcohol cleaned slides with a sable brush. Smears were heat-fixed at 50 °C for five minutes and stained for acetylcholinesterase.

Acetylcholinesterase staining. Acetylcholinesterase (AChE) activity was detected using the method of Karnovsky and Roots,¹⁸ as modified by Jackson² and Long and Williams.¹⁹ Smears were incubated at 22 °C in the following medium, made freshly each day, for 6.0 to 6.5 hours: 0.5 mg/mL acetylthiocholine iodide (Sigma, St Louis), 75 mmol/L dibasic sodium phosphate (pH 6.0), 5 mmol/L sodium citrate, 3 mmol/L copper sulfate, and 0.5 mmol/L potassium ferricyanide. Slides were then gently rinsed with distilled H₂O and fixed for ten minutes in 95% ethanol. They were counterstained in Harris' hematoxylin for 30 seconds, rinsed gently in running tap H₂O, blued in saturated lithium carbonate, and rinsed a final time in H₂O. Cytochemical control smears were incubated in media lacking substrate and were negative for reaction product in all cells.

A recent study indicated that eosinophils may contain AChE when examined by phase microscopy.⁴ To examine the selectivity of the AChE marker, 100 μm sections of bone marrow fixed in 1.5% glutaraldehyde/1.25% paraformaldehyde in 0.1 mol/L cacodylate buffer were stained for AChE activity by the method of Lewis and Knight²⁰ using acetylthiocholine iodide as the substrate. Silver/silver-gold sections were examined with a EM10CA transmission electron microscope (Zeiss, West Germany).

Mapping and ^3H -TdR-labeling of small AChE-positive Cells. Counterstained smears were coverslipped and the positions of 200 to 250 SACHE cells/mouse were located on the slides. This procedure was necessary since processing the slides for autoradiography decolorizes the AChE reaction product (see below). SACHE cells were located with phase microscopy (Zeiss) under a 100 $\times$

oil-immersion objective and had to fit the following criteria: (a) a cell diameter of less than 18 μm (measured with a calibrated stage micrometer), since morphologically recognizable megakaryocytes were always greater than 18 μm ; (b) the presence of cytoplasmic AChE reaction product, which appears black and granular under phase microscopy and diffusely golden brown under bright field microscopy; and (c) a high nuclear-cytoplasmic ratio. The cell diameter and nuclear morphology of each SACHE cell was recorded. To map each cell, stage micrometer readings were noted, and the field was photographed.

After the cells for all 36 mice were mapped, the coverslips were removed from the slides, which were then cleaned in fresh xylene. The dried smears were dipped in NTB-2 film emulsion (Kodak) diluted 1:1 with distilled H₂O. After thorough drying the slides were exposed in light-tight boxes containing dessicant at 4 °C for 30 days. The slides were then developed in fresh D-19 diluted 1:1 with distilled H₂O for five minutes (with agitation every 30 seconds), rinsed in distilled H₂O for one minute, and fixed in undiluted Kodak fixer for five minutes. The entire developing procedure was carried out at 18 °C, and all slides were processed on the same day. It is this development procedure that causes decolorization of the AChE reaction product.² The slides were then rinsed in running H₂O for 15 minutes and stained with a Romanovsky stain.

Mapped SACHE cells were located on each slide using the stage micrometer readings to find the correct field and then the photograph to locate the reaction product-positive cell (now decolorized) within the field. For each SACHE cell, the number of grains over the nucleus was counted under a 100 $\times$ oil-immersion objective (Fig 1), and the nuclear morphology was recorded again.

Background counts were estimated in two ways: (a) grain counts were done for 500 cells/slide on ten slides treated identically to the experimental autoradiograms, except that the smears were made with bone marrow from mice that were not injected with ^3H -TdR; and (b) grain counts were done on the experimental autoradiograms over areas away from the cells. Background counts determined by both methods were low (mean grain count was less than one grain/cell). For a cell to be considered labeled, it had to have a grain count of at least three times the background count.

The grain count data were analyzed at each time point after administration of ^3H -TdR to calculate the LI (percentage of SACHE cells that were labeled) and the mean grain count of the labeled SACHE cells. The data were analyzed statistically using a three-way analysis of variance.

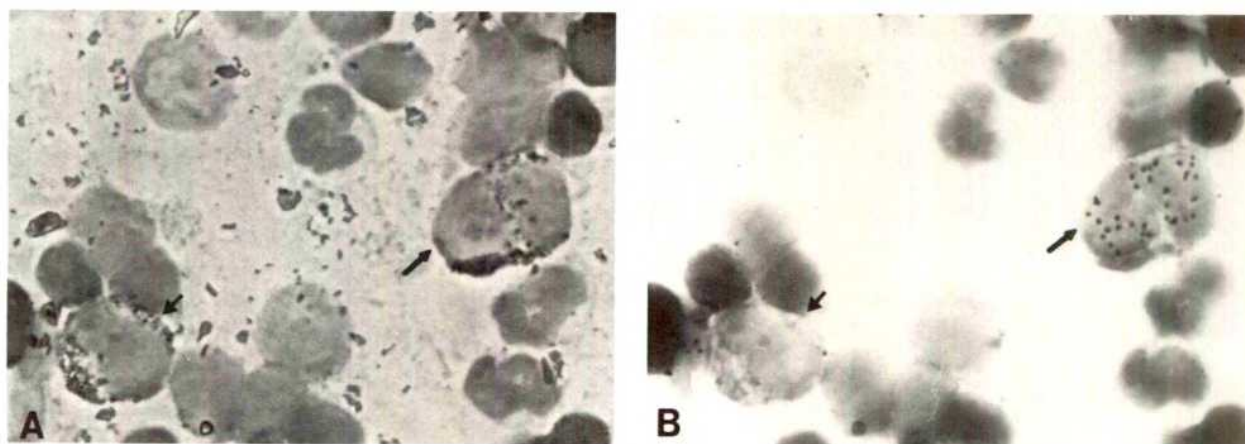


Fig 1. SACHE cells in bone marrow of a normal serum-treated mouse 30 minutes after administration of ^3H -TdR (phase contrast, original magnification 500 $\times$; current magnification 375 $\times$). (A) Note AChE reaction product in cytoplasm of SACHE cells (arrows). (B) Same field as (A) after processing for autoradiography. Note that the AChE reaction product is decolorized and that one SACHE cell with a lobulated nucleus has incorporated ^3H -TdR.

RESULTS

Platelet counts. Platelet counts are presented in Fig 2. For all experimental animals the platelet count was less than 5% of both the pretreatment and control values. Thereafter, the platelet count increased and reached pretreatment values by 76 hours after APS administration. NS had no effect on the platelet count.

Labeling index of SACHE cells. The labeling index (LI) for SACHE cells was defined as the percentage of SACHE cells that were labeled and was plotted against time after ^3H -TdR administration for experimental and control animals (Fig 3). The initial LI (at 30 minutes) for both groups was about 30% (29% for experimentals and 31% for controls). At four hours there was no change (29% for experimentals and 32% for controls), but by 12 hours the LI reached about 50% in both groups (52% in experimentals and 51% in controls). For the next three time points experimental and control values differed. At 24 hours the LI was 42% for experimental animals and 69% for controls. Both groups attained peak values at 48 hours (77% in experimentals and 92% in controls). By 72 hours there was a decline in LI for both groups (54% in experimentals and 76% in controls). The difference between experimental and control groups was significant ($P < 0.02$).

Mean grain counts. The mean grain count (MGC) for each animal was calculated by averaging the grain counts of all the labeled cells for that animal. For comparison, the MGC at 30 minutes was assigned a value of 1.0, and each subsequent MGC was expressed as a proportion of the initial value. These data are presented in Fig 4. Both groups demonstrated a rise in MGC at four hours, and the MGC decreased at each subsequent time point. The difference in MGC between groups was not significant ($P > 0.1$).

To summarize, in both experimental and control groups, the LI increased after four hours, peaked at 48 hours, and then decreased. After 12 hours the LI for the experimental group was lower than for the control group. In both groups the MGC decreased sharply at first and thereafter at a slower rate.

Morphology of SACHE cells. The nuclear morphology of SACHE cells was sometimes difficult to assess with

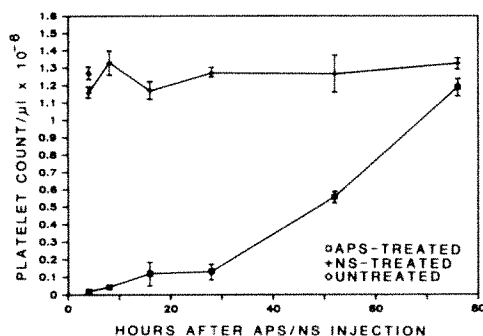


Fig 2. Mean platelet counts of mice after administration of antiplatelet serum (APS) or normal serum (NS). The mean platelet count of 26 untreated mice is shown on the graph for comparison. Error bars indicate ± 1 standard error of the mean (SEM). Where error bar is absent, SEM falls within the point.

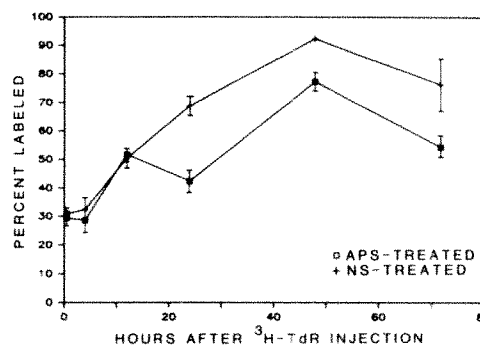


Fig 3. Labeling index of SACHE cells in APS- and NS-treated mice. Error bars indicate ± 1 SEM. Where error bar is absent, SEM falls within the point.

certainly, as round and tightly lobulated nuclei could not always be distinguished. If the nuclear morphology could be determined unequivocally, the cell was included in the analysis. The three morphologic types described by Long and Williams¹⁹ were noted in the present study, although the percentages were slightly different. Examples of SACHE cells before and after autoradiographic processing are presented in Fig 1.

In APS-treated mice, there was an altered frequency distribution of nuclear morphologies that varied with time (Table 1). As indicated by these data, experimental mice had decreased percentages of cells with a round and indented nucleus and an increased percentage of cells with a lobulated nucleus at early time points. Later on the percentage of cells with a round or indented nucleus was increased along with a decrease in the proportion of cells with a lobulated nucleus ($P < 0.05$).

The nuclear morphology of cells initially labeled was compared to that of unlabeled cells (Table 2). The frequency distribution of nuclear morphologies of both labeled and unlabeled cells was similar to that of all SACHE cells at 30 minutes after ^3H -TdR or 4.5 hours after APS administration, indicating that uptake of ^3H -TdR was not favored by a

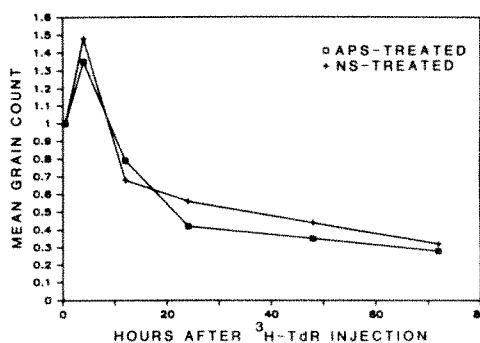


Fig 4. Relative mean grain count of SACHE cells in APS- and NS-treated mice. A value of 1.0 was assigned to the mean grain count (MGC) at 0.5 hour, and each subsequent MGC was expressed as a proportion of the initial value. The actual values ± 1 SEM for experimental and control animals respectively were: at 30 minutes, 22 ± 1 and 25 ± 2 ; at 4 hours, 30 ± 5 and 37 ± 5 ; at 12 hours, 18 ± 2 and 17 ± 3 ; at 24 hours, 9 ± 1 and 14 ± 1 ; at 48 hours, 8 ± 0 and 11 ± 1 ; and at 72 hours, 6 ± 0 and 8 ± 2 .

Table 1. Nuclear Morphology of SACHE Cells

Time after APS (h)	n	Round	Indented	Lobulated
4.5	976	1.35 $\pm$ 0.02	6.50 $\pm$ 0.07	92.14 $\pm$ 0.08
8	663	4.79 $\pm$ 0.08	11.65 $\pm$ 0.14	83.56 $\pm$ 0.22
16	683	3.91 $\pm$ 0.08	10.92 $\pm$ 0.13	85.17 $\pm$ 0.20
28	560	1.96 $\pm$ 0.05	12.39 $\pm$ 1.80	85.60 $\pm$ 0.08
52	664	9.27 $\pm$ 0.06	21.68 $\pm$ 0.21	69.05 $\pm$ 0.21
76	623	9.35 $\pm$ 0.06	22.58 $\pm$ 0.15	68.07 $\pm$ 0.14
Control	3,849	5.06 $\pm$ 0.03	16.81 $\pm$ 0.08	78.13 $\pm$ 0.11

Values are mean percent $\pm$ SEM; n, number of SACHE cells evaluated.

cell with a particular nuclear morphology. In APS-treated mice, the percentage of labeled cells with a round nucleus showed a greater increase with time than that of unlabeled cells. By 72 hours after ^3H -TdR administration, 12.15% $\pm$ 0.09% had a round nucleus (n = 337; mean $\pm$ SEM) as compared to 5.54% $\pm$ 0.39% (n = 286) in unlabeled cells and 9.35% $\pm$ 0.10% (n = 623) in all SACHE cells from APS-treated mice at 72 hours ($P < 0.02$).

SACHE cells were subject to both shrinkage and swelling due to the effects of fixation, histochemical reactions, and autoradiographic processing. Cell diameters could not, therefore, be considered absolute measures of cell size and were more useful for relative comparisons among groups of cells. The mean diameter of SACHE cells from APS-treated animals was 12.59 $\pm$ 0.23 μm (n = 4,609; mean $\pm$ SD) and for NS-treated animals was 12.63 $\pm$ 0.18 μm (n = 4,387; mean $\pm$ SD). There were no significant differences between time points or treatment groups ($P > 0.1$). In APS-treated mice the cell diameter of initially labeled cells was significantly greater than that of unlabeled cells (14.24 $\pm$ 1.69 μm and 11.96 $\pm$ 1.59 μm , respectively; $P < 0.001$). Results from control animals were similar.

Electron microscopy. The AChE reaction product was present in mature megakaryocytes and in a population of small cells but was absent in eosinophils at all stages of maturation (Fig 5).

DISCUSSION

The following conclusions can be made from the present study: (a) SACHE cells are heterogeneous with respect to cell cycle, with only 30% initially incorporating ^3H -TdR. The percentage increases with time, peaking at 48 hours, but is lower in APS-treated animals after 12 hours. The MGC decreases concomitantly with the increasing LI. (b) SACHE cells are heterogeneous with respect to nuclear morphology, and the lobulated nucleus is the most prevalent form, followed by the indented and round forms. Following injection of APS, the percentages of round and indented forms

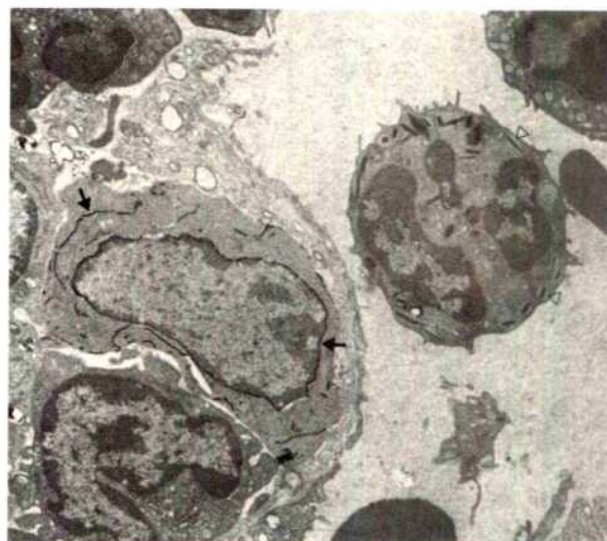


Fig 5. Electron micrograph of bone marrow stained for AChE activity (original magnification 7200 $\times$; current magnification 5400 $\times$). An AChE-positive cell contains reaction product in the nuclear envelope and in profiles of endoplasmic reticulum (arrows). An eosinophil with characteristic crystalloid inclusions (arrow heads) contains no reaction product.

decrease below control values but are higher than control values after two days. Cells with all three nuclear morphologies are capable of incorporating ^3H -TdR, and the percentage of cells with a round nucleus is greater in labeled than in unlabeled cells by 72 hours. (c) While the size of cells in the compartment of SACHE cells remains stable, cells that initially incorporate ^3H -TdR are larger than unlabeled cells.

We had hypothesized that a high proportion of SACHE cells would incorporate ^3H -TdR based on the increased LI of mature megakaryocytes by 24 hours^{6,7,21} and the apparent susceptibility of precursors of mature megakaryocytes to cytotoxic drugs.^{22,23} Since only 30% of SACHE cells initially labeled in this study, the possibility that mature megakaryocytes have a more immediate precursor than the SACHE cell was considered. This is unlikely in view of the studies on density profiles²⁴ and the relationship of SACHE cells to megakaryocytes.²⁵ A more likely explanation for the initially low labeling index is that SACHE cells are heterogeneous with respect to cell cycle status.

The lower peak LI in thrombocytopenic animals is probably the result of both an influx of cells from an initially unlabeled compartment and an efflux of labeled cells into the compartment of recognizable megakaryocytes, with both of these events occurring at a more rapid rate in thrombocytopenic animals. Under conditions of acute thrombocytopenia similar to those in the present experiments, the increase in

Table 2. Nuclear Morphology of Labeled and Unlabeled SACHE Cells 30 Minutes Post- ^3H -TdR Administration

	Labeled				Unlabeled			
	n	Round	Indented	Lobulated	n	Round	Indented	Lobulated
APS-treated	275	1.58 $\pm$ 0.08	5.26 $\pm$ 0.23	93.17 $\pm$ 0.22	701	1.31 $\pm$ 0.02	6.98 $\pm$ 0.10	91.72 $\pm$ 0.09
NS-treated	252	4.09 $\pm$ 0.17	14.39 $\pm$ 0.80	81.53 $\pm$ 0.76	683	3.45 $\pm$ 0.13	11.49 $\pm$ 0.14	85.06 $\pm$ 0.24

Values are mean percent $\pm$ SEM; n, number of SACHE cells evaluated.

CFU-M in S phase occurred by 24 hours and reached a maximum value of 49% at 40 hours.¹⁴ While a slight increase occurred at 12 hours, no change was observed at six hours. Thus, the majority of CFU-M would be unlabeled by ³H-TdR administered four hours after APS. As these unlabeled cells begin to synthesize DNA, they would eventually acquire the AChE marker and would enter the SACHE cell compartment. These unlabeled cells would decrease the LI. In control animals, influx of unlabeled cells may also occur, but at a slower rate. Efflux of labeled SACHE cells into the compartment of recognizable megakaryocytes is also likely to occur at a more rapid rate in thrombocytopenic animals, as labeled cells appear in the compartment of Stage I megakaryocytes more rapidly in thrombocytopenic animals.^{11,12}

Antibody-induced damage of SACHE cells is an unlikely cause for the lower peak LI in experimental animals. Previous studies have suggested that damage to mature megakaryocytes is minimal or lacking when small single doses of antiserum are used.^{16,17,26-28} In addition, administration of thrombopoietin or APS had similar effects on SACHE cells with respect to changes in the percentage of these cells in the bone marrow.³ Also, no reduction in CFU-M occurred when bone marrow cells were preincubated with APS prior to plating or when APS was included in the plating mixture.¹⁴

The decrease in LI at 72 hours in both groups of mice can be explained by the maturation of labeled SACHE cells into recognizable megakaryocytes and the entry of unlabeled cells into the SACHE cell compartment by 72 hours. This is consistent with reports that recognizable megakaryocytes attain peak labeling at three days due to an influx of labeled precursors and maturation of cells through the compartment.^{6,7}

In both experimental and control groups in this study, the MGC decreased while the LI increased. Cellular division, resulting in the distribution of labeled DNA among an increased number of cells, is one explanation for this phenomenon. However, while some SACHE cells may be capable of cellular division, most are not proliferating^{25,29} but are undergoing endomitosis. Other factors that may have contributed to the concurrent increasing LI and decreasing MGC are efflux of more heavily labeled SACHE cells into the compartment of recognizable megakaryocytes and influx of lightly labeled cells from the CFU-M compartment. Further studies are needed to explore these various possibilities.

Consistent with a previous report,¹⁹ we found that the most frequent nuclear morphology is the lobulated form. If this is also the most mature SACHE cell, the higher percentage present four to 24 hours after APS may represent a "shift to the right" in this population of cells. This is also suggested by the larger size of initially labeled cells.

In an earlier study,³⁰ hydroxyurea, an inhibitor of DNA

synthesis that lethally damages cells in S phase,³¹ was found to reduce only SACHE cells with a round nucleus, which represented less than 2% of the total SACHE cell compartment.¹⁹ Our studies indicate that uptake of ³H-TdR, which labels cells in DNA synthesis but is not cytotoxic at the doses used,³² is not limited to cells with a round nucleus. In fact, the highest percentage of cells that initially label have a lobulated nucleus. The marked difference in the mechanisms of action of these agents makes comparison difficult; however, the discrepancy in the percentage and morphology of cells affected may be the result of a higher resistance of polyploid SACHE cells, which incorporate ³H-TdR during endomitosis, to the lethal effects of hydroxyurea.

Recently a question was raised about the positive staining of eosinophils at the light microscopic level.⁴ In the present study it is unlikely that the AChE-positive cells represent eosinophils for the following reasons: (a) eosinophils that have a lobulated or segmented nucleus are postmitotic and therefore would not incorporate ³H-TdR; (b) cytoplasmic reaction product was visualized as black and granular with phase microscopy and diffusely golden brown (Hatchett's brown) under bright field microscopy; after decolorization of the AChE reaction product, no eosinophilic granules were seen in the Romanovsky-stained smears; and (c) in the examination of marrow sections by electron microscopy, we found that with the more sensitive technique of ultrastructural localization of AChE reaction product, positive eosinophils were never found at any stage of maturation.

These data are consistent with the following model of megakaryocytopoiesis: the CFU-M, normally in a relatively quiescent state, replenishes its own pool while feeding the SACHE cell compartment. SACHE cells are a heterogeneous population of cells with respect to cell cycle status and morphology, and are immediately responsive to marked changes in platelet mass. At least some proportion of SACHE cells are capable of cellular proliferation, while the most mature have begun the process of endomitosis. This compartment of cells is replenished by the CFU-M, and this occurs at a more rapid rate after the stress of thrombocytopenia. In turn, SACHE cells feed the compartment of recognizable megakaryocytes as the most mature SACHE cells, which are larger and have a lobulated nucleus, become Stage I megakaryocytes. Differentiation of cells committed to megakaryocytopoiesis may be directed by local and systemic mechanisms. SACHE cells constitute a heterogeneous group of cells in which the key element of differentiation is the change from mitosis to endomitosis, regulated at least in part by the end product of mature megakaryocytes.

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Erythroid Marrow Function in Anemic Patients

By Mario Cazzola, Pensri Pootrakul, Helmut A. Huebers, Mary Eng, Joseph Eschbach, and Clement A. Finch

Erythropoietic activity is known to be closely associated with marrow iron uptake. A modification of the standard measure of plasma iron turnover has been developed in which erythron transferrin uptake (ETU) rather than iron uptake has been calculated. The ETU has the advantage of providing a parameter of erythroid marrow activity independent of change produced by plasma iron and transferrin saturation. Measurements in 80 patients with anemia were compared to the normal value of 60 ± 12 $\mu\text{mol/L}$ whole blood/d. The mean ETU for ten patients with severe aplastic anemia and for six patients with pure red-cell aplasia were 12 ± 8 and 12 ± 11 $\mu\text{mol/L}$ whole blood/d, respectively. In ten transfusion-dependent patients with renal failure under dialysis therapy, the mean value was 35 ± 11 , while ten other dialyzed patients who were transfusion independent had a mean ETU of 73 ± 21

$\mu\text{mol/L}$ whole blood/d. Sixteen patients with hemolytic anemia had an average ETU of 400 ± 130 , while 28 patients with ineffective erythropoiesis had a mean value of 474 ± 147 $\mu\text{mol/L}$ whole blood/d. While patients with hypoproliferative anemia showed no relation between the severity of anemia and ETU, those with hyperproliferative erythroid marrow showed increasing values as the anemia became more severe. Sequential measurements in patients with aplastic anemia under treatment and in thalassemic patients under transfusion therapy showed the value of this measurement in monitoring the effects of treatment on erythroid marrow activity. It is concluded that the measurement of ETU provides a more direct ferrokinetic evaluation of erythroid activity in anemic states.

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DISORDERS of erythroid marrow function may be characterized as affecting marrow proliferation and/or red cell maturation. For clinical purposes the former is evaluated by marrow aspiration or biopsy, and the latter by the relationship between the reticulocyte index and marrow proliferation as judged by marrow examination.¹ Limitations to the quantitation of erythroid marrow activity from a minute sample have led to a continued search for a more adequate means of estimating erythroid marrow activity. Beginning with the original measurements of Huff and associates,² the measurement of plasma iron turnover has been continually refined to reflect more quantitatively erythron iron uptake.³⁻⁷

In the last few years, attention has focused on the reaction between transferrin iron and its membrane receptors.^{8,9} The assumption of a single plasma iron pool, so essential to previous kinetic measurements, has been shown to be erroneous.¹⁰ There are actually two plasma iron pools, one composed of monoferric transferrin and the other of diferric

transferrin. The diferric molecule has a greater capacity to deliver iron to tissue receptors than the monoferric species.^{9,11} Since the in vivo loading of iron on transferrin is a random phenomenon, the quantitative relationship between these two plasma iron pools and their respective iron clearance is predictable.¹⁰⁻¹² The critical reaction is the uptake of the transferrin-iron-receptor complex into the cell, and this occurs at the same rate, regardless of whether there are one or two molecules of iron on the transferrin molecule. In a recent study dealing with normal subjects, a simple approach to the measurement of transferrin-receptor interaction in normal man was presented, and the transferrin uptake was shown to be independent of plasma iron concentration and transferrin saturation.¹³ This approach has now been extended to the measurement of the erythron transferrin uptake in anemic patients.

MATERIALS AND METHODS

Patients. Ferrokinetic studies were performed in a total of 80 patients selected to provide extremes in erythropoiesis. A first group included ten individuals with severe aplastic anemia (AA) and six with pure red-cell aplasia (PRCA). Criteria for the diagnosis of severe aplastic anemia¹⁴ included a hypocellular bone marrow on bone marrow biopsy (less than 30% cellularity) and two of the following three factors: reticulocyte index <1 , granulocytes $<0.5 \times 10^9/\text{L}$, and platelets $<20 \times 10^9/\text{L}$.¹⁴ Criteria for the diagnosis of PRCA¹⁵ included the virtual absence of erythroid cells in the marrow but abundant marrow precursors for other cell species, a transfusion-dependent anemia associated with a decrease in reticulocyte index below 1, the presence of circulating granulocytes $>2 \times 10^9/\text{L}$, and platelets $>100 \times 10^9/\text{L}$. In addition to the above criteria and in order to select the most severely affected individuals, only those with less than or equal to 10% of injected radioiron in circulating red cells at two weeks were included in this first group. A second group was composed of 20 patients with severe renal disease with azotemia, all of whom were on renal dialysis. Within this group ten patients needed repeated transfusions to maintain their hematocrit over 20%, and ten other patients did not require transfusion.

The remaining 44 patients had anemia associated with erythroid marrow hyperplasia. There were 16 patients with hemolytic anemia, eight of whom had sickle cell disease, four hereditary spherocytosis, two red cell enzyme defects (pyruvate kinase deficiency, glucose 6-phosphate isomerase deficiency), one autoimmune hemolytic ane-

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mia, and one fragmentation hemolysis. Another group of 28 patients demonstrated ineffective erythropoiesis with erythroid marrow hyperplasia. Ten of these patients had β -thalassemia intermedia with hemoglobin concentrations between 6 and 9 g/dL,¹⁶ while 16 patients had β -thalassemia/Hemoglobin E disease, the criteria for which have been described elsewhere.¹⁷

Ferroketic measurements. Details of the measurement of plasma iron turnover have been previously summarized.^{18,19} In these studies it was standard practice to inject $^{59}\text{FeSO}_4$ (0.2 μg containing 2 μCi ^{59}Fe at pH 2) intravenously (IV) over a period of five minutes. However, in individuals with transferrin saturation >70%, radioiron was bound to normal plasma in vitro, and then the tracer saturation was adjusted to that of the patient's own plasma using cold ferrous ammonium sulfate.¹³

Plasma iron turnover was routinely calculated employing the formula¹⁹:

$$\text{PIT (mg/dL whole blood/d)} = \frac{\text{PI } (\mu\text{g/dL}) \times (100 - \text{Hct} \times 0.9)}{\text{T}_{1/2} \text{ (min)} \times 100}$$

This formula is suitable for turnover measurements as long as the blood volume (BV) is not significantly different from that predicted ($\pm 10\%$). A comparison was made of the determined BV based on the dilution of the injected isotope, as compared to the predicted BV.²⁰ Where there was a significant increase (>10%), the observed PIT was corrected by multiplying it by the ratio between determined and predicted plasma volume for the patient's plasmacrit.¹⁹

In some instances the radioiron red-cell utilization (RCU) was calculated from the red cell activity at 14 days and the amount of radioactivity injected as described by Cook and Finch.¹⁹

Additional calculations, discussed more in detail elsewhere,¹³ were made to convert the plasma iron turnover to the transferrin-iron complex uptake by the erythron. This involved (a) the subtraction of the extravascular plasma flux (EVF), (b) a correction designed to convert tissue iron uptake (IU) to tissue transferrin uptake (TU), and (c) the subtraction of nonerythroid transferrin uptake to leave erythron transferrin uptake (ETU).

$$\text{EVF (mg/dL wb/d)} = \text{PI } (\mu\text{g/dL}) \times \frac{(100 - \text{Hct} \times 0.9)}{100} \times 0.0015$$

$$\text{IU (mg/dL wb/d)} = \text{PIT} - \text{EVF}$$

$$\text{TU } (\mu\text{mol/L wb/d}) = \frac{\text{IU (mg/dL wb/d)} \times 10,000}{56} \times \frac{200 + 2.2S}{200 + 6.4S}$$

where S is the percent transferrin saturation, and wb/d stands for whole blood/day.

The final correction to allow for transferrin-iron going to non-erythroid receptors is made by subtracting the mean volume value of 11 $\mu\text{mol/L}$ whole blood/day¹³ from the calculated transferrin uptake:

$$\text{ETU } (\mu\text{mol/L wb/d}) = \text{TU } (\mu\text{mol/L wb/d}) - 11$$

Plasma iron and transferrin saturation were measured as described elsewhere.^{21,22} Hematocrit was determined by the micro-method. ^{59}Fe activity was determined by gamma counting. Plasma volume was calculated from the ^{59}Fe -transferrin dilution.¹⁹

Statistical analysis. Statistical analysis was performed using the CLINFO computer system (University of Washington, Seattle, WA). All results are given as mean $\pm$ 1 SD. The significance of the differences between means was tested by the Student's *t* test.

RESULTS

Data obtained in 80 subjects are summarized in Table 1. Ten patients with aplastic anemia had an average transferrin

saturation of $90\% \pm 9\%$, a mean plasma iron turnover of 0.57 ± 0.10 mg/dL whole blood/d, and a mean ETU of 12 ± 8 $\mu\text{mol/L}$ whole blood/d. Radioactivity in the blood at two weeks amounted to an average of $4\% \pm 4\%$ (range 0% to 10%). Six additional patients with PRCA had a mean transferrin saturation of $94\% \pm 5\%$, a mean plasma iron turnover of 0.57 ± 0.15 mg/dL whole blood/d, an ETU of 12 ± 11 $\mu\text{mol/L}$ whole blood/d, and a mean red-cell utilization at two weeks of $3\% \pm 3\%$ (range 0% to 6%). Thus, in these subjects the mean values for PIT were 80% of normal whereas ETU values averaged 20% of normal.

Ten patients with transfusion-dependent renal failure under continuous dialysis treatment had a mean transferrin saturation of $80\% \pm 15\%$, a mean plasma iron turnover of 0.73 ± 0.16 mg/dL whole blood/d, and an average ETU of 35 ± 11 $\mu\text{mol/L}$ whole blood/d. Red cell utilization in the ten patients averaged $26\% \pm 10\%$ (range 12% to 47%). By contrast, a second group of dialyzed patients who were able to sustain their hematocrit without transfusion had a mean transferrin saturation of $28\% \pm 6\%$, a mean PIT of $0.77\% \pm 18\%$ mg/dL whole blood/d, and a mean ETU of 73 ± 21 $\mu\text{mol/L}$ whole blood/d. Their red cell utilization averaged $71\% \pm 13\%$ (range 55% to 89%). Whereas there was no difference between the mean PITs of the two groups of renal patients ($t = 0.55$, $P > 0.05$), the mean ETU of those patients who did not need blood transfusions was significantly higher than that of transfusion-dependent patients ($t = 5.17$, $P < 0.0001$).

Sixteen patients with hemolytic anemia were heterogeneous in respect to the cause of their anemia, to its degree (hematocrit varied from 18% to 35.5%), plasma iron concentration (46 to 279 $\mu\text{g/dL}$), and transferrin saturation (11% to 91%). Plasma iron turnover averaged 3.86 ± 1.45 mg/dL whole blood/d but ranged from 1.26 to 6.64 mg/dL whole blood/d. ETU averaged 400 ± 130 $\mu\text{mol/L}$ whole blood/d but ranged from 168 to 612 $\mu\text{mol/L}$ whole blood/d.

A similar heterogeneity was observed in 28 patients with ineffective erythropoiesis. Hematocrits varied from 15.1% to 34.5%, plasma iron from 52 to 308 $\mu\text{g/dL}$, and transferrin saturation from 16% to 98%. Mean PIT was 5.11 ± 1.85 mg/dL whole blood/d with a range from 1.40 to 9.23, and ETU values averaged 474 ± 147 $\mu\text{mol/L}$ whole blood/d with a range from 176 to 809.

All patients with aplastic anemia, pure red-cell aplasia, and renal failure associated with anemia had erythroid marrow activity as judged by ETU between 0 and $1.6 \times$ basal. On the other hand, patients with hemolytic anemia and ineffective erythropoiesis showed values between 2.8 and 13.5 times basal (all values but two being greater than 3). When ETU was plotted against the hematocrit (Fig 1), there was no apparent relationship among hypoproliferative patients between the severity of the anemia and ETU ($r = 0.32$, $P = 0.06$), whereas there was an inverse correlation among hyperproliferative patients ($r = -0.71$, $P < 0.001$).

Sequential studies were carried out in five patients with pure red-cell aplasia under treatment after an appropriate interval of time (Fig 2). These measurements showed no change in marrow activity in two patients and improvement in three by both improvement in hematocrit and clearly

Table 1. Ferrokinetic Studies

Condition	Hct (%)	Retic Index ($\times$ basal)	Plasma Iron (μ g/dL)	Tf Sat. (%)	T-1/2 (min.)	RCU (%)	PIT (mg/dL/d)	ETU (μ mol/L/d)
Normal Subjects (53)	42 $\pm$ 4	$\sim$ 1	112 $\pm$ 43	35 $\pm$ 11	80 $\pm$ 15	85 $\pm$ 4	0.71 $\pm$ 0.17	60 $\pm$ 12
Aplastic Anemia (10)	30	0.3	493	98	530	6	0.68	1
	22	0.0	319	96	415	0	0.62	10
	22	0.0	154	87	292	—	0.42	11
	13	0.5	189	80	272	—	0.61	23
	31	0.1	350	94	426	—	0.59	8
	24	0.3	197	69	404	0	0.38	4
	17	0.3	243	95	307	2	0.67	21
	17	0.1	282	95	464	0	0.51	3
	27	0.1	282	96	345	10	0.62	16
	25	0.1	228	90	307	8	0.57	17
Average:	23 $\pm$ 6	0.2 $\pm$ 0.2	274 $\pm$ 9	90 $\pm$ 9	376 $\pm$ 88	4 $\pm$ 4	0.57 $\pm$ 0.10	12 $\pm$ 8
Pure Red-Cell Aplasia (6)	14	0.0	202	85	399	6	0.44	6
	16	0.0	274	97	331	1	0.71	21
	27	0.2	300	94	245	—	0.77	28
	20	0.1	322	98	456	5	0.58	5
	29	0.0	178	94	275	—	0.47	14
	20	0.0	244	96	472	0	0.42	0
Average:	21 $\pm$ 6	0.1 $\pm$ 0.1	253 $\pm$ 56	94 $\pm$ 5	363 $\pm$ 94	3 $\pm$ 3	0.57 $\pm$ 0.15	12 $\pm$ 11
Renal Failure A (10)	28	1.2	146	50	206	47	0.53	28
	26	0.6	205	78	234	16	0.67	30
	22	0.8	217	82	249	33	0.70	30
	23	1.0	196	81	192	17	0.70	33
	26	1.0	261	89	202	23	0.99	52
	20	1.9	275	93	240	24	0.94	44
	20	2.4	181	85	222	32	0.67	31
	22	0.9	206	92	374	12	0.55	17
	18	1.6	187	88	177	33	0.88	48
	22	0.4	184	57	222	27	0.67	35
Average:	23 $\pm$ 3	1.2 $\pm$ 0.6	206 $\pm$ 38	80 $\pm$ 15	232 $\pm$ 55	26 $\pm$ 10	0.73 $\pm$ 0.16	35 $\pm$ 11
Renal Failure B (10)	29	1.6	82	30	70	85	0.87	83
	28	0.9	57	21	53	89	0.80	86
	31	1.4	76	28	94	71	0.58	50
	20	0.4	67	31	120	66	0.46	34
	24	2.0	135	41	114	62	0.93	75
	30	1.1	78	23	62	89	0.92	97
	16	2.8	56	22	84	57	0.57	54
	29	1.9	61	24	66	75	0.69	68
	20	0.8	84	34	76	55	0.91	84
	31	1.8	98	30	72	64	0.99	96
Average:	26 $\pm$ 5	1.5 $\pm$ 0.7	79 $\pm$ 24	28 $\pm$ 6	81 $\pm$ 22	71 $\pm$ 13	0.77 $\pm$ 0.18	73 $\pm$ 21
Hemolytic Anemia (16)	30	—	130	44	32	—	2.98	301
	24	—	72	30	19	—	3.74	432
	30	—	109	34	39	—	2.76	295
	26	9.0	148	60	25	66	4.56	435
	21	—	97	35	28	—	5.09	558
	21	—	104	38	23	—	5.65	612
	24	—	107	38	23	49	4.45	480
	22	—	279	91	34	—	6.64	545
	28	—	77	27	26	—	2.22	254
	32	—	254	81	32	—	5.52	482
	25	4.2	55	17	15	87	2.93	382
	32	—	46	11	26	95	1.26	168
	27	6.6	88	33	17	—	3.99	450
	34	4.2	133	40	44	—	2.09	209
	36	4.4	234	86	40	63	3.98	335
	18	6.0	78	34	17	—	3.89	435
Average:	27 $\pm$ 5	5.7 $\pm$ 1.9	126 $\pm$ 70	44 $\pm$ 24	27 $\pm$ 9	72 $\pm$ 19	3.86 $\pm$ 1.45	400 $\pm$ 130

Table 1. Ferrokinetic Studies (Cont'd)

Condition	Hct (%)	Retic Index ($\times$ basal)	Plasma Iron (μ g/dL)	Tf Sat. (%)	T-1/2 (min.)	RCU (%)	PIT (mg/dL/d)	ETU (μ mol/L/d)
Ineffective Erythropoiesis (28)	30	0.3	52	16	29	42	1.40	176
	27	0.3	94	35	21	28	3.38	371
	25	2.1	85	37	16	29	4.67	515
	22	0.8	80	39	25	41	4.55	486
	25	—	133	90	17	14	5.99	523
	23	—	128	86	25	19	6.24	545
	21	0.3	308	97	63	33	4.92	389
	20	1.4	73	35	13	22	5.63	635
	31	0.8	95	43	25	43	3.32	345
	24	—	60	34	14	23	3.86	434
	32	0.5	80	34	36	—	2.15	228
	15	—	134	91	18	9	9.23	809
	25	—	246	97	31	42	5.46	454
	19	—	241	93	37	28	6.56	551
	21	—	185	94	22	28	8.80	760
	20	—	280	85	40	44	8.44	726
	23	—	179	97	26	—	6.78	575
	18	—	243	98	47	—	5.53	450
	24	0.8	192	71	30	—	5.01	454
	25	1.2	137	59	24	—	4.45	426
	21	1.0	140	67	25	—	4.65	430
	30	0.8	138	49	30	—	3.37	336
	35	1.0	150	50	28	—	3.69	365
	31	0.5	139	51	23	—	4.36	435
	24	1.2	224	74	41	—	4.28	375
	24	1.4	207	74	23	—	7.03	642
	31	0.7	174	78	23	—	5.43	487
	31	1.2	179	67	33	—	3.93	358
Average:	25 $\pm$ 5	0.9 $\pm$ 0.5	156 $\pm$ 68	66 $\pm$ 25	28 $\pm$ 11	30 $\pm$ 11	5.11 $\pm$ 1.85	474 $\pm$ 147

demonstrable increases in marrow erythroid activity. Sequential measurements in patients with thalassemia or sickle cell anemia undergoing transfusion therapy showed the suppressive effect of higher hematocrit on erythroid activity but erythropoietic activity was still two to three times basal for hematocrit values around 40% (Fig 3). In these latter studies there was a close correlation ($r = 0.86$, $P <$

0.01) between ETU and erythroid: myeloid ratio of the marrow.

DISCUSSION

Within the circulating blood there is a constant flow of iron from donating cells via transferrin to membrane transferrin receptors of tissues requiring iron. The marrow normally receives about four fifths of all iron passing through

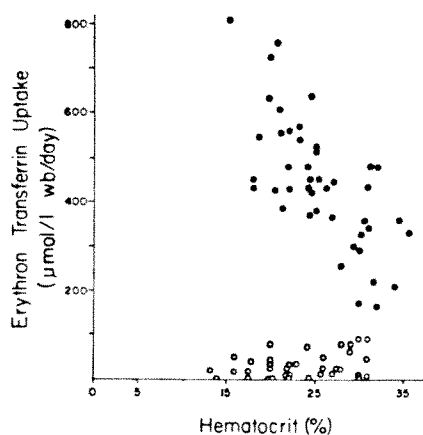


Fig 1. Relationship between hematocrit and erythron transferrin uptake (ETU) in anemic patients. Patients with conditions known to be associated with impaired proliferative response to anemia are shown with open circles (○) and patients with conditions associated with a normal proliferative response to anemia are shown with solid circles (●).

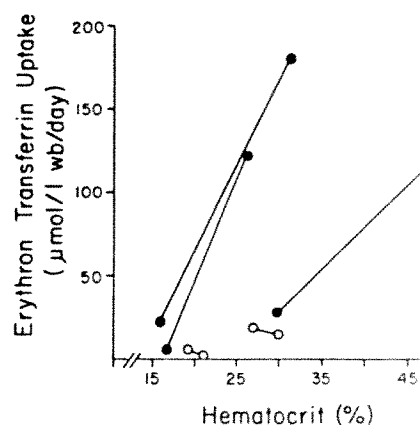


Fig 2. Ferrokinetic studies carried out before and after treatment in patients with pure red cell aplasia. Three patients showed both improvement in hematocrit and increased marrow erythroid activity, while two unresponsive patients showed no change in either.

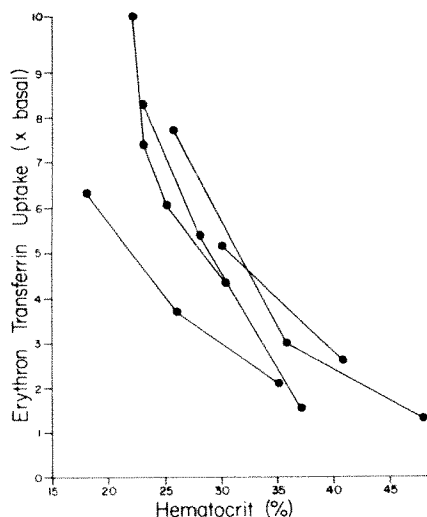


Fig 3. Sequential ferrokinetic studies in patients with thalassemia and sickle cell anemia undergoing transfusion therapy. The relationship between hematocrit concentration and erythropoietic activity is illustrated.

the plasma for incorporation into hemoglobin. It has therefore been assumed that erythroid marrow function would be quantitatively related to iron turnover. In some conditions, however, the plasma iron turnover gives a poor reflection of the state of erythropoiesis, since other pathways of iron exchange are increased while erythron iron uptake is reduced. A number of elegant models of iron kinetics have been developed that take into consideration the amount of iron going to erythroid and nonerythroid tissues and various refluxes of iron from those tissues.^{4,6,7} Such models require sophisticated processing of experimental data derived from blood sampling over ten to 14 days, and although they have been of value in developing an understanding of erythroid disorders, they are not practical for clinical usage and the results are themselves subject to criticism. These models are based on the erroneous assumption of a single plasma iron pool and, for the most part, have not considered the effect of transferrin saturation on iron turnover. The principal focus of this work was to develop a simple ferrokinetic approach to quantitation of erythropoiesis by applying recent knowledge concerning iron exchange between transferrin and body cells to ferrokinetic measurements.

The plasma iron turnover is derived from the hematocrit, the plasma iron, and the rate of disappearance of radioiron from circulation.^{18,19} Before a relationship between iron turnover and erythropoietic activity could be exploited, it was first necessary to allow for transferrin iron going to extravascular fluid and not initially taken up by cells. This was done based on the relationship previously described by Cook et al between plasma iron and extravascular flux.⁴

The key to the present approach depends on the reaction between transferrin iron and its membrane receptors. We have previously shown that the plasma iron turnover increases with increasing plasma iron and transferrin saturation.¹³ Cook et al⁴ thought this was due to increased nonerythroid iron uptake, an assumption no longer tenable. It is now known to be due to an increased proportion of diferric iron as

saturation increases and the greater capacity of diferric transferrin to deliver iron to all membrane receptors, erythroid and nonerythroid. This does not necessarily imply that erythropoiesis is increased at higher saturations but is rather a reflection of the increased proportion of diferric transferrin molecules.

We have demonstrated that transferrin uptake can be derived empirically from plasma iron turnover, once extravascular flux has been subtracted, knowing the preference ratio between the two forms of plasma iron and the relative amount of each.¹³ Transferrin uptake so derived was found to be independent of plasma iron and transferrin saturation, and this suggested that the critical measurement of erythroid marrow function could be best described by erythroid transferrin uptake once allowance had been made for nonerythroid transferrin uptake. The nonerythroid tissues of the body have a limited capacity to assimilate iron. After injection of radioiron, normal subjects have only about 15% of the activity outside of the red cell mass at two weeks. Furthermore, there is little evidence to date that these nonerythroid receptors, most of which reside in the liver, can change greatly in number except in the presence of iron deficiency (Tanin et al, unpublished data). This means that the number of iron-bearing transferrin molecules reacting with membrane receptors on nonerythroid tissues can be considered relatively constant, and the mean normal value of 11 μmol transferrin/L whole blood/d has been assumed in the present study.¹³ According to these calculations, erythron transferrin uptake in normal subjects averages 60 ± 13 μmol /L whole blood/d.

A first opportunity to examine the appropriateness of the various corrections made to obtain the ETU was provided by patients with severe aplastic anemia and pure red-cell aplasia. Among the 16 patients studied, whose plasma iron turnover ranged from 0.38 to 0.77 mg/dL whole blood/d, values for ETU ranged from 0 to 28 μmol /L whole blood/d. No patient had a value below 0, indicating that estimates of nonerythroid iron turnover were not overcorrected. The red cell utilization of 3% to 4% when compared to the mean ETU of 12% the basal value suggested that a major portion of the residual erythroid activity in these patients did not result in the production of viable red cells. Evidence of dyspoiesis in aplastic anemia has been reported.^{23,24}

Studies in renal patients provided another opportunity to compare the clinical status with the ferrokinetic evaluation of erythropoiesis. Renal patients have a combination of impaired marrow stimulation due primarily to inadequate erythropoietin production and also increased red cell hemolysis, but erythropoiesis is effective.¹⁸ Studies of two groups of dialyzed patients, one self-sufficient and the other transfusion dependent, showed no difference in plasma iron turnover (0.77 ± 0.18 v 0.73 ± 0.16 mg/dL whole blood/d) but a conspicuous difference in erythroid activity in the two groups, ie, 73 ± 21 v 35 ± 11 μmol /L whole blood/d. Assuming that both had the same degree of shortening of red cell life span to about one-half normal, the difference in hemoglobin concentration was adequately explained by the greater impairment in production in the transfused group.

Patients with hemolytic anemia had an average ETU of 400 ± 130 , and patients with ineffective erythropoiesis $474 \pm$

147 $\mu\text{mol/L}$ whole blood/d. These can be translated into mean erythropoietic rates of 6.7 and 7.9 times basal, respectively. In some of these patients the reduction of erythropoiesis after transfusion was measured (Fig 3). Quantitative aspects of the relationship between blood hemoglobin concentration or hematocrit and suppression of erythropoiesis are not well defined. There is ample clinical evidence that suppression of erythropoiesis by transfusion is required for thalassemic patients if distension of the marrow and distortion of the skeleton are to be prevented. Previous measurements on patients with thalassemia were made by Cavill et al²⁵ at hemoglobin levels from 9 to 17 g/dL. According to their detailed analysis of the plasma iron disappearance curve, erythropoiesis as judged by marrow iron turnover became normal or subnormal at a blood hemoglobin concentration of 11 to 12 g/dL. They comment, however, that the marrow still showed normoblastic hyperplasia. It might be anticipated from the type of analysis performed by them that ineffective erythropoiesis could not be completely separated from iron exchange with nonerythroid tissues, and this could cause total erythropoiesis to be underestimated.^{18,26} In studies reported here, erythropoiesis did decrease as the hemoglobin concentration was increased by transfusion but remained

two to three times basal at a hemoglobin concentration of 11 to 12 g/dL, and parallel changes in erythroid: myeloid ratio validated the ferrokinetic measurement. Evaluation of erythroid activity can be of value in determining the degree of erythroid suppression after transfusion in individual patients with thalassemia major and also in deciding whether patients with thalassemia intermedia should be transfused.

Taken as a group, individuals with an impaired marrow response to anemia, ie, less than two times basal, showed no relationship between the ETU and hematocrit, the slope of the regression line being not significantly different from 0. This suggested that the impairment in production was the dominant feature in causing the anemia and that the erythroid marrow could not respond appropriately to the erythropoietin stimulus produced by anemia. In contrast, patients with an appropriate proliferation response to anemia showed an inverse relationship between hemoglobin concentration and erythropoietic response. This would be consistent with an increased marrow stimulation by erythropoietin as the hemoglobin fell.¹ A similar relationship has been observed employing a more complex ferrokinetic approach.²⁷

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cis-Retinoic Acid Stimulates the Clonal Growth of Some Myeloid Leukemia Cells In Vitro

By H. Jeffrey Lawrence, Kevin Conner, Michael A. Kelly, Mark R. Haussler, Paul Wallace, and Grover C. Bagby, Jr

We studied the effects of cis-retinoic acid (cisRA) on the clonogenic growth of samples of leukemic cells from 35 patients with acute nonlymphocytic leukemia (ANLL). We observed significant inhibition of leukemic colony growth in 17 samples by 10^{-7} to 10^{-6} M cisRA. However, we found that retinoid exposure resulted in striking stimulation of clonal growth in ten samples at the same drug concentrations. With the exception of cases with promyelocytic features, there was no morphologic or functional evidence that cisRA induced the leukemic blasts to differentiate. Both inhibition and stimulation were dose-dependent and observable at pharmacologically achievable levels of cisRA. Leukemic cells with monocytic features more frequently demonstrated a stimulatory response than did those without monocytic features. Depletion of T lymphocytes and monocytes did not alter the type of growth response.

THERE HAS BEEN substantial interest in the use of retinoids in the chemoprevention of cancer and as noncytotoxic chemotherapeutic agents in malignancy. Numerous studies in animal models have demonstrated the capacity of vitamin A and its derivatives to prevent malignant progression in various preneoplastic epithelial lesions and even reverse overt carcinoma.¹ Retinoids have also been shown to be potent inducers of granulocytic differentiation in a human promyelocytic leukemia cell line, HL-60,² and in blasts from patients with acute promyelocytic leukemia.³ Retinoids have been reported to inhibit the clonal growth of myeloid leukemic cells in vitro at pharmacologically achievable levels⁴ and to enhance the growth of normal myeloid and erythroid progenitors.^{5,6} These data suggest that retinoids might have clinical utility in the treatment of acute nonlymphoblastic leukemia (ANLL) by inhibiting the growth of the malignant clone, by enhancing normal stem cell proliferation, and, in some cases, by inducing terminal differentiation in the leukemic blasts. Because of the small numbers of cases of ANLL previously studied in vitro, we undertook an investigation of the effects of retinoic acid on the clonal growth and differentiation of leukemic cells from a larger group of patients and attempted to elucidate the mechanisms of action.

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Assays for cellular retinoic acid-binding protein (CRABP) were performed on five samples (two with inhibitory growth responses, two with stimulatory responses, and one with no growth) and failed to reveal detectable levels of CRABP in any case. The addition of cisRA to liquid suspensions of leukemic cells produced no significant change in the number of viable cells. We conclude that the effects of cisRA on leukemic colony growth are not cytotoxic and not mediated by T lymphocytes, monocytes, or CRABP. More importantly, cisRA appears to enhance the growth of certain human leukemia cells in vitro. Taking into account the increasing use of retinoids in clinical trials for patients with leukemia, the latter findings may represent a significant cautionary note.

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METHODS

Patients. Table 1 outlines clinical characteristics of the 35 patients studied. A breakdown by the French-American-British (FAB) classification shows a predominance of cases with FAB M-2 and M-4 morphology.⁷ Two cases of acute promyelocytic leukemia were studied. Most patients had received no prior therapy. The large number of patients with a previous history of a preleukemic condition (ten cases of myelodysplasia, one case of polycythemia rubra vera, and one case of Down's syndrome) reflects the link between our research interests and referral patterns. All patients involved in this study were advised of the methods and possible risks of marrow aspiration, in accordance with institutional guidelines, and gave informed consent.

Retinoic acid. 13-Cis-retinoic acid (cisRA) was kindly provided by Hoffman-LaRoche (Nutley, NJ) and was suspended in absolute alcohol at an initial stock concentration of 10^{-2} M and serially diluted in phosphate-buffered saline (PBS) to provide concentrations of 10^{-4} to 10^{-9} M. Control solutions containing ethanol alone were also prepared. CisRA and the controls were stored at -70°C in foil-wrapped tubes and were added to cultures under subdued light.

Leukemic cells. Heparinized samples of bone marrow or peripheral blood were obtained and light-density mononuclear cells isolated by a standard centrifugation step with Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ).^{8,9} In studies on the effect of T lymphocyte depletion, T cells were removed by rosetting with sheep erythrocytes (Prepared Media Laboratories, Tualatin, OR) and a second Ficoll-Hypaque separation.^{9,10} Monocyte depletion was accomplished by incubating cell suspensions in alpha medium and 10% fetal calf serum in large flat-bottomed flasks for 60 to 90 minutes at 37°C in a humidified 5% carbon dioxide atmosphere. Nonadherent cells were then removed by gentle pipetting and resuspended in their original volume.¹⁰

Blast colony assay. Leukemic blast colonies were grown using a modification of the methods of Minden et al¹¹ and Buick et al.¹² Agar underlayers were prepared in advance with 10% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM, see below) and varying concentrations of cisRA or ethanol. Leukemic cells were suspended in 0.9% methylcellulose with alpha medium and 20% fetal calf serum at 100,000 to 200,000 cells/mL and plated in 1 mL aliquots on each agar underlayer. Plates were wrapped loosely in foil and incubated at 37°C in a humidified 5% carbon dioxide atmosphere for seven days. Aggregates of five or more cells were counted as blast colonies and expressed as numbers per 100,000

Table 1. Patient Population

Number	35
Adults/Children	31/4
Age	1-85 yr
	Median 58
Male/Female	25/10
FAB Class	
M-1	5
M-2	14
M-3	2
M-4	11
M-5	1
M-6	2
Previously untreated/treated	27/8
History of preleukemic disorder	12

cells plated. Three or four replicates were placed for each variable. A difference between variables was considered significant if the *P* value using a Student's paired *t* test was less than 0.05.

PHA-LCM. Buffy coat leukocytes were harvested from phlebotomy specimens of patients being treated for idiopathic hemochromatosis and placed in liquid suspension at 10^6 cells/mL with RPMI, 10% fetal calf serum, and 1% vol/vol phytohemagglutinin (Burlington Wellcome, Research Triangle Park, NC) by the method of Aye et al.¹³ Cell suspensions were incubated at 37 °C in a humidified 5% carbon dioxide atmosphere for three to five days. Supernatants were harvested and stored at 4 °C until used. Batches were screened for stimulatory activity, and samples from two donors were used for all studies because of their potency.

Assay for cytoplasmic retinoic acid-binding protein (CRABP).¹⁴⁻¹⁶ Cryopreserved leukemic cells from five patients (75 to 200 × 10⁶ cells per case) were rapidly thawed in a 37 °C water bath and washed twice in PBS, sonicated in KET-O buffer (1 mmol/L EDTA/10 mmol/L Tris-HCl, pH 7.4), and centrifuged at 159,000 × *g* for 45 minutes to obtain a supernatant (cytosol) fraction. The cytosols were first divided and diluted with KET-O or 6 mmol/L pCMBS (Sigma, St. Louis, MO) in KET-O to a final pCMBS concentration of 2 mmol/L and incubated for 30 minutes at 4 °C to block pCMBS-sensitive ligand binding to CRABP. The diluted cytosols were then evaluated for CRABP content as follows. Cells from the 3T6 murine fibroblast line were used as a positive control. For qualitative identification of specific retinoic acid binding to CRABP, 0.4 mL of diluted cytosol was incubated 22 to 26 hours in the dark at 4 °C with 50 nmol/L 35.9 Ci nmol [³H] retinoic acid (Hoffman-LaRoche, Nutley, NJ) alone or in the presence of a 200-fold excess of 0.01 mmol/L nonradioactive retinoic acid alone or in the presence of a 200-fold excess nonradioactive retinoic acid alone. Incubates were then analyzed by centrifugation on 4.6 mL 5% to 20% sucrose gradients at 234,000 × *g* for 22 hours. Radioactivity in five-drop fractions taken from the top of the gradients was counted in 5 mL of ACS scintillation cocktail (Amersham Corp, Arlington Heights, IL) at an efficiency of 26%. Sedimentation markers used were lactoglobulin (2.0 S), ovalbumin (3.7 S), and bovine serum albumin (4.4 S). For quantitative analysis, 100 μL aliquots of diluted cytosol or pCMBS-treated cytosol was then incubated for 24 hours at 4 °C with 0 to 100 nM [³H] retinoic acid, with the former set of incubations yielding total binding and the latter giving a measure of nonspecific binding. All points were run in duplicate and bound, and free retinoic acid was separated by adding 1 mL of 2.5% dextran T-70-coated charcoal in gelatin/phosphate buffer (0.15 mol/L NaCl/0.015 mol/L NaN₃/0.1 mol/L Na₂HPO₄/0.039 mol/L NaH₂PO₄, pH 7.03/0.1% gelatin). After 15 minutes, the charcoal suspension was centrifuged at 4,000 × *g* for 10 minutes and radioactivity in the supernatant was counted in 10 mL

of ACS cocktail (26% efficiency). In all cases cytosolic protein was determined by the method of Lowry et al.¹⁷

Studies of differentiation. Cytospin preparations were made from samples of leukemic blasts grown in liquid suspension (11 samples) or from leukemic colonies plucked from semisolid medium (three samples) with and without cis-retinoic acid 10⁻⁶ mol/L. Slides were stained with Wright-Giemsa stain (Camco Quik Stain, Cambridge Chemical Products, Ft. Lauderdale, FL). Differential counts were performed on 100 to 200 cells per slide. Cells were categorized by standard morphologic criteria either as primitive (blasts, promyelocytes, promonocytes) or as mature (myelocyte stage and beyond).

Nitro blue tetrazolium (NBT) reduction test was performed using standard methodology with phorbol myristate acetate (Sigma, St. Louis, MO) and Ficoll.¹⁸ After counterstaining with nuclear fast red, differential counts were performed on 100 to 200 cells per slide. Cells containing fine blue-black granules were scored as positive, and the percentage of positive cells was determined. A positive control using peripheral blood leukocytes from a normal donor was prepared with each batch of leukemic samples studied.

RESULTS

Blast colony assays. Clonal assays were attempted from 35 samples of leukemic blasts from patients with ANLL. Five samples showed no growth. Clonal efficiency varied widely with colony counts ranging from 0 to 1,920 per 100,000 plated cells. The median number of colonies was 89 (mean, 486). Unstained blast colonies had a characteristic morphology, forming very tight and usually spherical aggregates at the agar/methylcellulose interface. The average number of cells per aggregate was 10 to 20, although in some colonies the numbers exceeded 100. Wright staining of cytocentrifuge preparations or of air-dried colonies revealed aggregates of primitive cells with large nuclei with fine nuclear chromatin, prominent nucleoli, occasional cytoplasmic granules, and vacuoles without evidence of terminal differentiation. The cells within the colonies did not form rosettes with sheep erythrocytes.

Effects of retinoic acid on clonal growth. In three cases cisRA had no significant effect on clonal growth at concentrations of 10⁻⁷ to 10⁻⁶ mol/L. In 17 cases significant inhibition of colony growth was observed with cisRA at levels of 10⁻⁷ and 10⁻⁶ mol/L, both pharmacologically achievable levels (Table 2A). Inhibition ranged from 22% to 100% of the appropriate ethanol-containing controls with a median decrease of 69%. Inhibition increased with dose in eight of ten samples tested at multiple levels.

In ten cases we observed striking stimulation of colony growth ranging from 83% to 1500% of controls with a median increase of 176% (Table 2B). The control colony growth (medium of 129 and mean of 450 colonies per 100,000 plated cells) of these ten cases was not significantly different from the group as a whole. In seven cases the stimulation of growth increased with dose of cisRA. Figure 1 demonstrates a representative dose-response curve for a patient with enhanced colony growth. Ethanol had no effect on clonal growth except for the highest dose tested, whereas cisRA was strikingly stimulatory at levels of 10⁻⁷ – 10⁻⁵ mol/L. When T lymphocytes were removed before culture (lines with open circles), the effects of cisRA were essentially unchanged.

Table 2A. Effects of Cis-Retinoic Acid on Leukemic Colony Growth in Samples With Inhibitory Responses

Patient Number	CisRA 10^{-6} mol/L*	Percent Difference†	CisRA 10^{-7} mol/L*	Percent Difference†
1	950/1428	-39%	ND	—
2	21/89	-76	46/109	-58%
3	32/565	-94	276/469	-41
4	ND	—	0/1029	-100
5	457/1053	-57	204/857	-76
6	ND	—	7/17	-59
7	122/519	-76	ND	—
8	13/16.5	-21	ND	—
9	2844/4306	-34	2804/4652	-40
10	0/91	-100	117/85	+38‡
11	22/44	-50	ND	—
12	70/153	-54	97/153	-37
13	32/120	-73	68/120	-43
14	9/29	-69	12/29	-59
15	137/238	-42	220/205	+7‡
16	ND	—	0/5	-100
17	47/240	-80	64/240	-73
Median percent decrease		-63%		-58%

*Numerator represents mean number of colonies/100,000 cells plated in presence of cis-retinoic acid (CisRA). Denominator represents mean colony numbers in control dishes. All colony counts represent means of three to four replicates.

†Percentage difference was calculated by subtracting control colony growth from CisRA-treated colony growth and dividing by control colony growth.

‡Not significant by Student's *t* test ($P > 0.05$). All other differences have $P < 0.05$. ND, not done.

We studied the effects of T cell depletion in three additional cases, two with inhibitory responses and one with a stimulatory response, and found that the removal of T cells did not change the type of response in any case (data not shown). Two samples, one from a patient with an inhibitory response and one with a stimulatory response, were used to study the effects of monocyte depletion. In the former case, cisRA decreased colony growth from 38 to 20 colonies per 100,000 plated cells in the unfractionated portion and from

Table 2B. Effects of Cis-Retinoic Acid on Leukemic Colony Growth in Samples With Stimulating Response

Patient Number	CisRA 10^{-6} mol/L*	Percent Difference†	CisRA 10^{-7} mol/L*	Percent Difference†
18	171/62	+176%	208/89	+134%
19	97/7	+1286	43/17	+153
20	ND	—	768/420	+83
21	1822/1572	+16‡	2421/1382	+84
22	6860/1556	+341	6676/1669	+300
23	133/59	+125	127/55	+131
24	330/170	+94	150/170	-12‡
25	64/4	+1500	52/4	+1200
26	2432/456	+433	2291/680	+237
27	152/90	+1590	160/11	+1354
Median percent increase		+341%		+144%

*See legend for Table 2A.

†See legend for Table 2A.

‡Not statistically significant by Student's *t* test ($P > 0.05$). ND, not done.

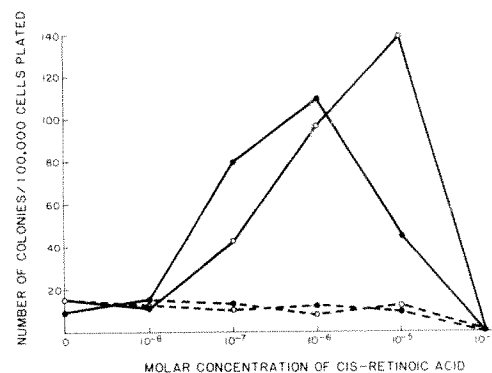


Fig 1. Effect of drug dose and T cell depletion on blast colony growth of cells from patient 19 with a stimulatory response. Results are expressed as absolute numbers of colonies per 100,000 plated cells. Light density cells grown in retinoic acid (●—●), light density cells grown in appropriate ethanol control media (○—○), T cell depleted cells grown in retinoic acid (○—○), T cell depleted cells grown in appropriate ethanol control media (○—○).

40 to 25 colonies/100,000 plated cells in the monocyte-depleted portion. In the latter case cisRA increased colony growth from four to 19 colonies/100,000 cells in the unfractionated portion and from four to 27 colonies/100,000 cells in the monocyte-depleted portion. Thus, monocyte depletion by an adherence procedure did not alter baseline blast colony growth nor the effect of cisRA.

Of the 17 patients with inhibitory responses to cisRA, only four had acute myelomonocytic leukemia (FAB-M4). Of the ten patients with a stimulatory response, seven had monocytic features. It appeared that cases with monocytic features more frequently displayed stimulatory responses than did other subtypes of leukemia ($P < .05$). Among the 12 samples from patients with a previous preleukemic condition, six were inhibited and five were stimulated by cisRA, and one sample showed no growth. Retinoid acid was inhibitory to colony growth in one case of acute promyelocytic leukemia and had no significant effect in the others.

CRABP assay. Cells from five patients, including two with stimulatory responses, two with inhibitory responses, and one with no growth (patient 18) were assayed for the presence of CRABP. Four of the five were evaluated qualitatively by sucrose gradient technique and found to be negative, ie, no specific 2S binding (Fig 2). All five were studied quantitatively by saturation analysis employing pCMBS and were found to have no measurable pCMBS-sensitive binding, indicating less than 0.5 pmol of CRABP per mg protein in all samples tested. By contrast, cytosol from 3T6 cells yielded specific 2S binding, which was inhibited by pCMBS and by an excess of unlabeled cisRA (Fig 2). The final protein concentrations in the incubation mixtures ranged from 0.3 to 0.5 mg/mL with median of 0.44 mg/mL.

Studies of differentiation. Liquid suspension cultures were prepared from leukemic cells from 18 patients. Cells were grown in alpha medium, 20% fetal calf serum, and 10% PHA-LCM with and without 10^{-6} mol/L cisRA. After seven days incubated cells were harvested and cytospin preparations were made. Among 17 samples (ten with inhibitory

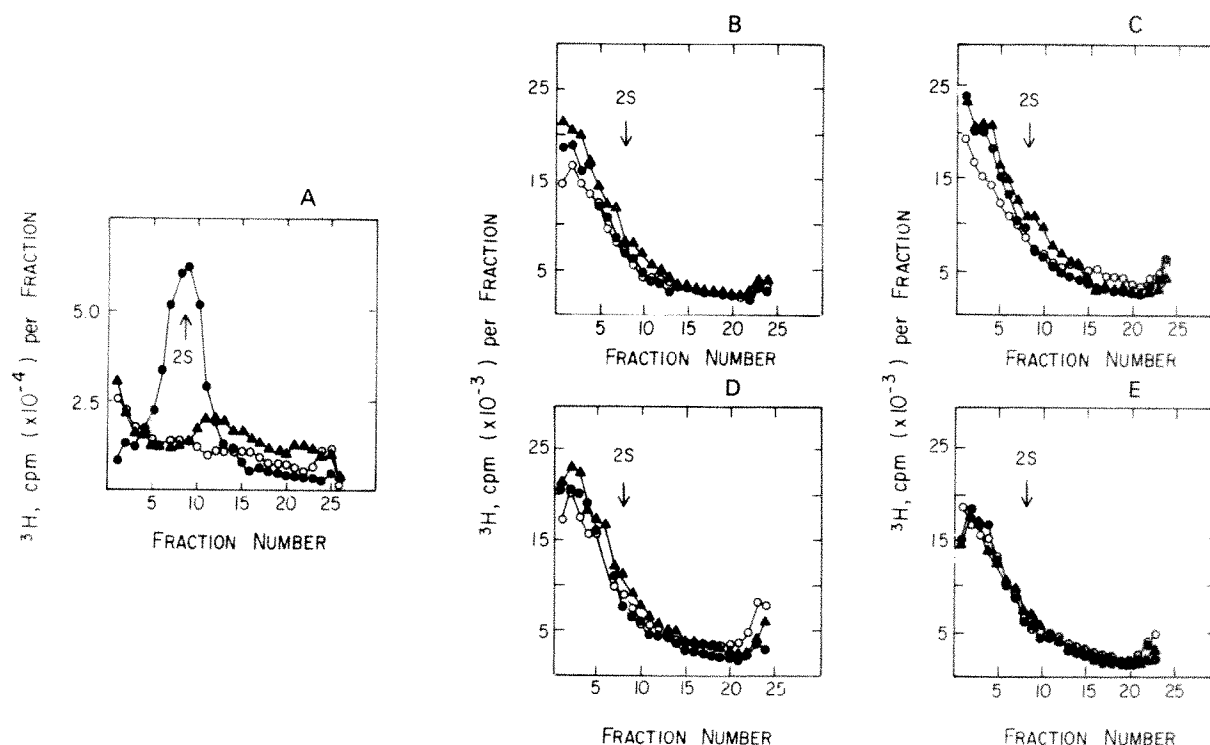


Fig 2. Sucrose gradient analysis of binding of [^{3} H]retinoic acid to cytosol of (A) 3T6 cells (B and C) patients 3 and 5 with inhibitory response, (D) patient 22 with a stimulatory response, and (E) patient 33, whose cells did not form colonies. Cytosols were incubated with 50 nmol/L [^{3} H]retinoic acid alone (●), with 50 nmol/L [^{3} H]retinoic acid plus a 200-fold excess of unlabeled retinoic acid (○), or with 50 nmol/L [^{3} H]retinoic acid after treatment with pCMBS (▲).

responses and seven with stimulatory response in clonogenic assay) there was no difference in the percentages of primitive cells in retinoid-treated (median 90%, range 75% to 97%) and untreated samples (median 93.5%, range 78% to 100%). By contrast, the cells from one patient with APL showed striking granulocyte differentiation with cisRA (only 3% primitive cells as compared with 82% in control flasks).

In three cases blast colonies were plucked from the methylcellulose cultures and stained for morphologic evaluation. In two samples, one showing growth inhibition and one showing stimulation, cisRA treatment produced only primitive colonies composed entirely of blasts. In one case of APL, nine/11 colonies plucked from dishes containing cisRA were composed of mature cells while 12/12 colonies from untreated dishes were comprised of promyelocytes.

NBT tests were performed on 16 samples of leukemic cells after seven days of culture in liquid suspension with and without 10^{-6} mol/L cisRA. As can be seen in Table 3, retinoic acid did not significantly increase NBT positivity in any sample except the promyelocytic cell line HL60. Thus, with the exception of HL60 cells and cells from patients with APL, no patient's cells showed morphologic or functional evidence of differentiation in response to cisRA.

To exclude a nonspecific cytotoxic effect of retinoic acid on leukemic blasts, the numbers of viable leukemic cells after exposure to 10^{-6} mol/L cisRA in liquid suspension were determined. Cell cultures were set up at an initial cell concentration of 2×10^5 cells/mL. After four and seven days of incubation, aliquots of cisRA-treated and untreated sam-

Table 3. Effects of Retinoic Acid on NBT Testing of Leukemic Cells

Samples	Control*	CisRA* 10^{-6} mol/L
Normal peripheral blood	95%-98%	—
HL-60 cells	5	71
Samples with inhibited colony growth		
Patient No.		
3	9	4
5	21	25
6	1	4
11	8	13
14	0	2
17	16	24
Samples with stimulated colony growth		
18	21	16
20	7	6
22	0	1
25	51	51
26	75	58
27	48	48
Samples with no growth		
32	3	5
34	32	31
35	19	20

*Percentage of NBT positive cells (100 to 200 cells scored/sample).

ples were counted for numbers of viable cells with a hemocytometer and trypan blue exclusion. After four days of culture, there was no difference in cell numbers between retinoid-treated and untreated cells ($3.2 \pm 1.4 \times 10^5$ cells/mL v $3.4 \pm 1.7 \times 10^5$ cells/mL, 17 samples). Similarly, after seven days cell numbers were virtually identical ($4.8 \pm 2.6 \times 10^5$ cells/mL with cisRA v $4.5 \pm 2.5 \times 10^5$ with control, ten samples). Clearly there was no evidence that cisRA was cytotoxic in short-term culture. Furthermore, changes in cell numbers in liquid suspension had no correlation with retinoid-induced changes in clonogenic growth.

DISCUSSION

The results of this study indicate that mononuclear cells from blood or marrow of patients with ANLL will form aggregates of blast-like cells in a majority of cases. The fact that the cells in these aggregates do not form E rosettes and that colony counts were not significantly altered by T lymphocyte depletion excludes the possibility that the colonies arise from T lymphocytes contaminating the specimens. The lack of morphologic or functional features of mature granulocytes or monocytes makes it highly unlikely that these colonies arise from residual normal myeloid progenitors (CFU-GM). Previous studies have failed to demonstrate in vitro growth of normal CFU-GM from leukemic marrows.^{19,20}

The data demonstrate that cisRA significantly inhibits blast colony growth in most samples (17/30) of leukemic cells showing growth under basal conditions and that inhibition occurs at levels of the drug that can be achieved pharmacologically in man.^{21,22} However, the stimulation of growth in ten samples suggests that in some cases the drug might have an adverse effect on patients, especially since the growth enhancement seen in our cases was much greater than the twofold increase reported for normal hematopoietic stem cells grown in the presence of cisRA.^{5,6}

It is important to note that we observed no morphologic or functional evidence that cisRA induced differentiation in the leukemic samples we studied, whether growth was enhanced or inhibited, except for the two cases of acute promyelocytic leukemia. These observations are entirely consistent with those of Breitman et al³ who noted no evidence of maturation in human leukemic cells incubated with retinoids unless the cells had promyelocytic features. Our findings are perhaps more significant than Breitman's in that our observations were made on leukemic cells grown in the presence of colony-stimulating factors while his cultures were unstimulated.

The mechanisms of action of cisRA on leukemic growth are unclear. The effects appear not to be mediated by monocytes or T lymphocytes. It seemed important to exclude

a possible mediating role of auxiliary cells, since (a) monocytes and T cells elaborate cytokines, which stimulate the growth of normal and leukemic hematopoietic stem cells^{10,23,24}; (b) T cells may develop cytotoxicity for autologous leukemic blasts^{25,26}; and (c) retinoids enhance the immune functions of T lymphocytes.^{27,28} It is still possible that the effects of cisRA on our freshly obtained leukemic cells are mediated by other auxiliary cells such as B lymphocytes.

CRABP, a 2S protein with high affinity and specificity for retinoic acid,²⁹ has been considered a likely candidate as a mediator of the effects of the vitamin, since CRABP has been detected in breast cancer cells, embryonal carcinoma cells, neuroblastoma cells, melanomas, retinoblastomas, and other tumor cell lines and has been associated with both the antiproliferative and differentiation-induction effects of retinoic acid in those tumors.^{15,30-31} Although one group has reported the detection of CRABP in HL-60 cells,³² other groups have not found CRABP in HL-60 or any other myeloid leukemic blasts; hence it seems unlikely that normal CRABP is a mediator of the effects of cisRA in these cells.^{4,33}

Another potential fruitful area of investigation is the study of the effects of retinoids on oncogene expression in malignant cells. Other groups have reported marked decreases in *c-myc* and *c-myb* mRNA in HL-60 cells exposed to retinoids.^{34,35} Our own preliminary studies suggest that the effects of retinoids on oncogene expression in freshly obtained leukemic cells are complex.³⁶ Since oncogenes may play a role in cell proliferation and on the expression of the malignant phenotype, altering oncogenic expression in leukemic cells may inhibit cell growth and/or promote differentiation.

In our experience, in vitro responses to certain pharmacologic agents, including glucocorticosteroids, are predictive of in vivo responses to the same agents.^{37,38} Clinical trials of cisRA in leukemia, preleukemia, and smoldering leukemia have already been undertaken.³⁹⁻⁴³ Based on the observations, we fear that in some patients acceleration of the leukemic process may occur. We believe that therapeutic trials must include prospective in vitro testing of the effects of retinoids on leukemic cell growth to determine if growth patterns are predictive of clinical responses and, further, that if adverse in vitro responses correlate with adverse clinical responses, retinoid therapy should be ruled out in patients whose leukemic cells proliferate in response to the agent in vitro.

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Proliferative and Differentiative Responses of B Cells From Human Marrow Graft Recipients to T Cell-Derived Factors

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Upon activation, B cells express growth and differentiation receptors that permit them to proliferate and differentiate. The aim of this study is to define the nature of the intrinsic B cell defects found in marrow recipients using assays for B cell activation, proliferation, and differentiation. B cells from five short-term (< three months postgrafting) and 15 long-term (> one year postgrafting) marrow recipients (ten with and five without chronic graft-v-host disease [GVHD]) were studied. T cell supernatants (T-sup) were prepared by stimulating normal T cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and phytohemagglutinin. Highly purified B cells were used to assess B cell proliferation responses to T-sup after *Staphylococcus aureus* Cowan I (SAC) activation and for B cell immunoglobulin production responses to T-sup stimulation after SAC activation. B cells from all five short-term patients and one long-term patient with chronic GVHD did not respond to

any stimulation. B cells from two patients with chronic GVHD responded to SAC but had decreased proliferative and differentiative responses to T-sup. B cells from three of seven patients with chronic GVHD and two of five long-term healthy patients could proliferate but could not secrete immunoglobulin in response to SAC plus T-sup stimulation. Furthermore, there was a significant correlation between serum IgG and/or IgM in marrow recipients and the differentiative responses of their B cells to T-sup ($P = 0.0075$, Fisher's Exact). B cell defects occur at various stages of maturation postgrafting. These defects include the failure to respond to the SAC activation signal, the failure to proliferate in response to T-sup, and the failure to differentiate in response to T-sup. These findings are probably due to the inability of B cells from certain marrow recipients to undergo a second round of ontogeny.

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RECENTLY it has been shown that B cell growth and differentiation is regulated by various lymphokines such as interleukin 1 (IL 1), interleukin 2 (IL 2), B cell growth factor (BCGF), B cell differentiation factor (BCDF), and the interferons.¹⁻⁸ Resting B cells can be activated by cross-linking of their immunoglobulin (Ig) molecules with multivalent ligands such as antigen, anti-Ig antibody, or protein A from *Staphylococcus aureus*. Upon activation, B cells express the receptors for BCGF and/or BCDF and, in the presence of T cell-derived factors containing BCGF and BCDF, go on to proliferate and terminally differentiate into Ig-secreting cells.⁹⁻¹¹

Marrow transplantation is being used for the treatment of hematologic malignancies, severe aplastic anemia, and some inborn errors with increasing success.¹²⁻¹⁵ However, marked immunodeficiencies associated with life-threatening opportunistic infections develop and may persist up to one to two years after grafting.^{16,17} Although numbers of T and B cells in the peripheral blood usually recover within one to three

months postgrafting, T and B cell collaboration is ineffective.¹⁸⁻²⁶ In vitro studies of marrow recipients using Ig biosynthesis stimulated with polyclonal activators revealed that there is functional heterogeneity within T cell subsets,^{23,24} normal monocyte function,²⁶ and intrinsic B cell defects in Ig production.⁹⁻²⁶ A recent study using an antigen-specific antibody production system showed that decreased antigen-specific B cell precursor frequency is one explanation for deficient antibody production in a minority of marrow recipients.²⁷ However, such data suggested that the majority of B cell defects were due to problems other than low antigen-specific B cell precursor frequency. These findings provided the rationale for studying B cells using a different approach.

Since nearly half of marrow recipients have B cells that failed to produce immunoglobulin, we chose assays for B cell proliferation and differentiation after *S aureus* Cowan I (SAC) activation that would identify the stages at which such defects occur. This investigation shows that B cells from marrow recipients have defects in SAC activation, defects in response to proliferative signals, and defects in response to differentiative signals.

METHODS

Patients. Twenty patients were studied between 63 and 1,736 days postgrafting. Five patients were studied at less than three months postgrafting (short-term), and 15 patients were studied more than one year postgrafting (long-term). Their unique patient number (UPN), age, sex, days of study after transplant, diagnosis, treatment at the time of testing, and serum Ig levels are presented in Table 1. These patients were conditioned for their marrow transplant as described.^{12,13,28} Nineteen patients received HLA-identical allogeneic marrow from their siblings. One patient (UPN 2590) received a syngeneic marrow graft. Posttransplant immunosuppression to prevent graft-v-host disease (GVHD) has been described.²⁸ The patient who received the syngeneic marrow graft did not receive postgrafting immunosuppression. Marrow engraftment was determined by red blood cell enzyme and antigen typing and by cytogenetic studies. All of these studies were done when the patients were clinically stable. Control subjects were healthy individuals or mar-

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Table 1. Patient Characteristics and Treatments

UPN	Age/Sex	Days post-Tx	Diagnosis	Treatment	Serum Ig (mg/dL)		
					IgG	IgM	IgA
Short-term recipients							
2879	37/F	84	CML	CS	1126	71	181
2909	17/F	69	ANL	CS	1114	119	<u>103</u>
2910	40/M	82	CML	CS	680	177	79
2948	18/F	69	AA	CS	725	<u>42</u>	58
2936	33/F	63	ANL	CS	854	72	58
Long-term recipients with chronic GVHD							
2461	23/F	399	ANL	P	<u>577</u>	<u>53</u>	80
2533	28/M	347	ANL	None	904	275	<u>52</u>
2416	35/F	461	AA	P	<u>476</u>	77	<u>20</u>
2474	30/M	385	ALL	None	1386	113	108
1690	43/F	1183	ANL	None	1761	77	208
2534	31/M	368	L	P	<u>623</u>	135	<u>20</u>
1531	30/M	1391	ALL	P	<u>555</u>	<u>53</u>	<u>36</u>
2569	15/M	341	ANL	P	1156	257	332
2503	34/M	417	CML	None	1507	253	176
2317	24/F	584	ANL	P	<u>397</u>	96	<u>20</u>
Long-term recipients without chronic GVHD							
2590	15/F	319	AA	None	1185	57	<u>55</u>
2504	12/F	378	ALL	None	1255	109	90
2525	26/F	341	AA	None	1576	101	<u>68</u>
1311	21/M	1736	L	None	1175	152	115
2599	37/F	347	CML	None	<u>665</u>	190	82

Normal ranges of serum Ig (mg/dL): IgG 670 to 1700; IgM 56 to 275; IgA 70 to 350. Underlined Ig values are below the normal ranges.

Abbreviations: UPN, unique patient number; CML, chronic myelogenous leukemia; CS, cyclosporine; ANL, acute nonlymphocytic leukemia; AA, aplastic anemia; ALL, acute lymphocytic leukemia; L, lymphoma; P, prednisone.

row donors. These studies were performed after informed consent was obtained under protocols approved by the Human Subjects Review Committees of the University of Washington and the Fred Hutchinson Cancer Research Center.

Cell separation. Peripheral blood lymphocytes (PBL) were obtained from 40 to 70 mL of whole heparinized blood by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Monocytes were removed from PBL by incubation for 30 minutes at room temperature with 5 mmol/L 1-leucine methyl ester (Sigma, St. Louis) in serum-free Mishell Dutton balanced salt solution.^{29,30} Monocyte-depleted PBL were then separated into T and non-T cell populations by rosetting with 2-aminoethylisothiuronium bromide (AET)-treated sheep erythrocytes.³¹ The non-T population was rosetted a second time with AET-treated sheep erythrocytes. The nonrosetting cells were further depleted of T cells by monoclonal antibody 35.1 (E rosette receptor antibody) and rabbit complement (Pel Freez, Roger, AR) to remove residual T cells. B cells obtained in this manner contained more than 72% B cells, less than 1% T cells, and less than 16% (mean = 5.0%) monocytes as determined by indirect immunofluorescence using monoclonal antibodies 299 (pan B), 35.1 (pan T), and 60.1 (antimonocyte), and fluorescein isocyanate-conjugated goat antimouse Ig (TAGO, Burlingame, CA). The monoclonal antibodies were gifts from Drs John Pesando and John Hansen at the Fred Hutchinson Cancer Research Center. These B cell-enriched fractions were designated B cells. The E rosetting cells (T cells) contained more than 95% E rosette-positive cells.

Generation of T cell supernatants containing BCGF and BCDF activity. Normal T cells at a concentration of 4×10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS were incubated with 5 ng/mL of 12-O-tetradecanoyl-phorbol-13-acetate (TPA, Sig-

ma, St. Louis) for two hours at 37 °C in 24 well plates (Catalog #1-43982, Nunc, Denmark). The T cells were then washed, resuspended in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin-streptomycin, 4 mmol/L L-glutamine, and 5×10^{-5} mol/L 2-mercaptoethanol, and cultured for three days in the presence of phytohemagglutinin (1:640 dilution of stock, PHA, Difco) at a cell concentration of 2×10^6 /mL. The cell-free supernatants prepared in this manner were pooled from five individuals and stored at 4 °C until use. The T cell supernatant pool (T-sup) was screened for Ig by enzyme-linked immunosorbent assay (ELISA) and found to be <20 ng/mL. The T-sup contained 158 U/mL of IL 2 activity defined by titration on a CTLL murine cell line in comparison to recombinant IL 2.

Assay for B cell proliferation response. The ability of purified B cells to respond to BCGF contained in T-sup was assessed by using SAC (Pansorbin 10% vol/vol; Calbiochem-Behring Corp, San Diego, CA) stimulation following a modification of the procedure described by Muraguchi and Fauci.¹⁰ In brief, 1×10^5 B cells from normal subjects or marrow recipients were cultured in 0.2 mL of culture medium in the presence of 0.005% vol/vol SAC for three days in round-bottom microtiter plates (NUNC, Denmark). Then the culture media were replaced with fresh media containing various percentages of T-sup for three days. The cells were pulsed with 1 μ Ci [³H]-thymidine (New England Nuclear, Boston) for the last four hours. Incorporation of [³H]-thymidine was measured by standard liquid scintillation-counting techniques after harvesting the cells with a Titertek cell harvester (Flow Laboratories, Rockville, MD). B cell proliferative responses were assessed by comparing them with that of nine normal controls. In an arbitrary decision based on a relationship to the clinical status of the patients and a natural distribution of patient results above and below 30% of the median value, proliferative responses less than 30% of the median value of proliferative responses of normal B cells were considered defective.

Assay for B cell differentiation response. One hundred thousand B cells from normal subjects or marrow recipients were cultured in round-bottom microtiter plates in the presence or absence of SAC (0.005% vol/vol) and/or various dilutions of T-sup as indicated for ten days. B cell differentiative responses to T-sup were assessed by measuring culture supernatants for Ig. Supernatants from triplicate cultures were pooled, and the concentration of accumulated Ig in the culture supernatants was determined by ELISA assays specific for human IgG, IgM, and IgA.^{32,33}

Immunoglobulin production by B cells from patients was compared with that of 9 normal controls. In an arbitrary decision based on a relationship to the clinical status of the patients and a natural distribution of patient results above and below 20% of the median value, immunoglobulin production responses less than 20% of the median value of immunoglobulin produced by normal B cells were considered defective.

RESULTS

Proliferative responses of normal B cells to T-sup. Experiments were initially undertaken to determine the BCGF and BCDF activities present in the T-sup. The representative experiment in Fig 1 shows that SAC-stimulated B cells from a single individual gave a maximal response on day 3 that fell to essentially background by day 6. When T-sup were added to such cultures on day 3, the SAC-activated proliferative responses of B cells were sustained through day 8. These data show that T-sup supports B cell proliferative responses. Figure 2 shows B cell proliferative responses as a function of the reciprocal dilution of T-sup. Similar results were obtained when B cells from marrow recipients were

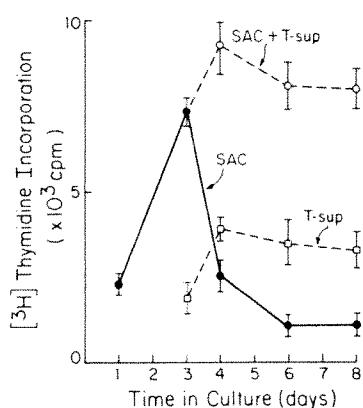


Fig 1. Kinetics of the proliferative response of B cells to SAC, T-sup, and SAC plus T-sup in a normal subject. One hundred thousand purified B cells were cultured with SAC (0.005 % vol/vol), T-sup (25%), or SAC plus T-sup for the indicated periods of time. SAC was added on day 0 and T-sup was added on day 3. The cultures were pulsed with [^3H]-thymidine four hours before harvest. The data points represent the mean $\pm$ 1.0 SD for triplicate cultures.

examined. From these data we chose a 1:4 dilution of T-sup for use in the B cell proliferation assay.

To exclude T cell contamination or proliferation during the culture, we examined the PHA-induced proliferative responses of B cells and the percentages of E rosette-positive cells at the end of cultures. The median proliferative responses of B cells to PHA stimulation from various study groups expressed as stimulation indices are 1.4 (range 0.3 to 2.4) in the normal control group; 0.8 (range 0.3 to 1.4) in short-term marrow recipients; 1.2 (range 0.4 to 1.8) in long-term recipients with chronic GVHD; and 1.2 (range 0.4 to 1.8) in long-term recipients without chronic GVHD. Only a small proportion of E rosette positive cells (0% to 2.0%)

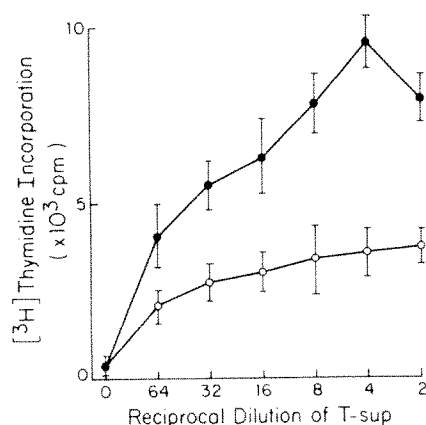


Fig 2. The relationship between the magnitude of B cell proliferation as a function of T-sup concentration in a normal subject. $\bullet$ = 100,000 B cells were cultured with SAC (0.005 % vol/vol) for three days and an additional three days with serial dilutions of T-sup. $\circ$ = 100,000 B cells were cultured with media for three days and an additional three days with serial dilutions of T-sup. The cultures were pulsed with [^3H]-thymidine four hours before harvest. The data points represent mean $\pm$ 1.0 SD of triplicate cultures.

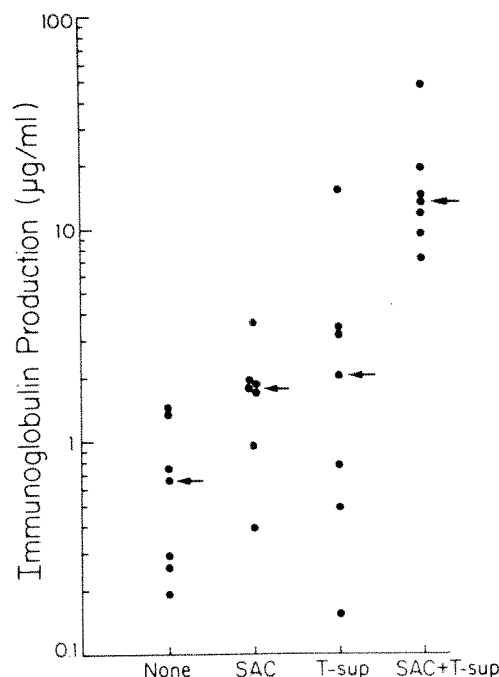


Fig 3. Effects of SAC, T-sup, and SAC plus T-sup on Ig production using purified B cells from seven normal subjects. One hundred thousand B cells were cultured with medium, SAC (0.005 % vol/vol), T-sup (0.03125 %), or SAC plus T-sup for ten days. Supernatants from triplicate cultures were pooled, and total Ig (IgG + IgM + IgA) produced was quantitated by ELISA. The median values of each group are indicated with ($\leftarrow$).

were present at the end of such cultures. Therefore, the proliferating cells were B cells and not contaminating T cells.

Differentiative responses of normal B cells to T-sup. B cell differentiation (Ig production) was assessed by SAC, T-sup, or SAC plus T-sup stimulation. The effects of T-sup on the differentiation of normal B cells were examined by quantitating Ig production in culture supernatants stimulated with SAC or T-sup alone or SAC plus T-sup. Figure 3 summarizes the results of seven separate experiments. Stimulation of B cells by SAC or T-sup alone occasionally induced detectable amounts of Ig in the culture supernatants. However, co-stimulation with SAC and T-sup consistently induced larger amounts of Ig than stimulation with SAC or T-sup alone. These results are similar to those reported in earlier studies.^{4,30}

In contrast to the B cell proliferation assay, the optimal concentrations of T-sup for Ig biosynthesis were much lower. Maximal Ig biosynthesis of IgG, IgM, or IgA occurred at a concentration of 3.125% (1:32). Higher or lower concentrations of T-sup in the cultures did not support Ig synthesis as well as the 1:32 dilution of T-sup (Fig 4). Similar results were obtained when recipient's B cells were examined. Therefore, a final concentration of 3.125% of T-sup was used for all subsequent B cell differentiation assays.

B cell proliferative responses to SAC in normals and marrow recipients. SAC-stimulated B cell proliferation responses are shown in Fig 5 for marrow recipients and control subjects. Purified B cells were stimulated with SAC

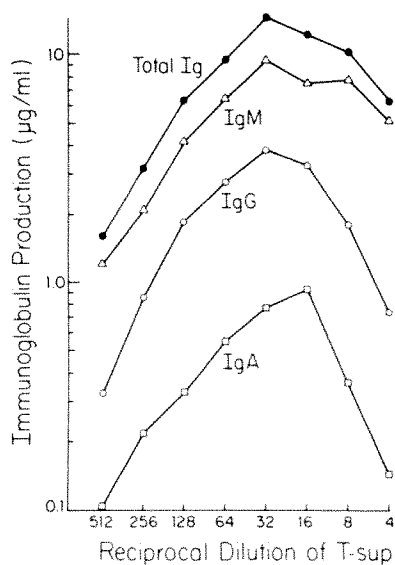


Fig 4. Immunoglobulin production by B cells from a normal subject as a function of the concentration of T-sup. One hundred thousand B cells were cultured with SAC (0.005 % vol/vol) and serial dilutions of T-sup for ten days. Supernatants from triplicate cultures were pooled and class-specific Ig production was quantitated by ELISA.

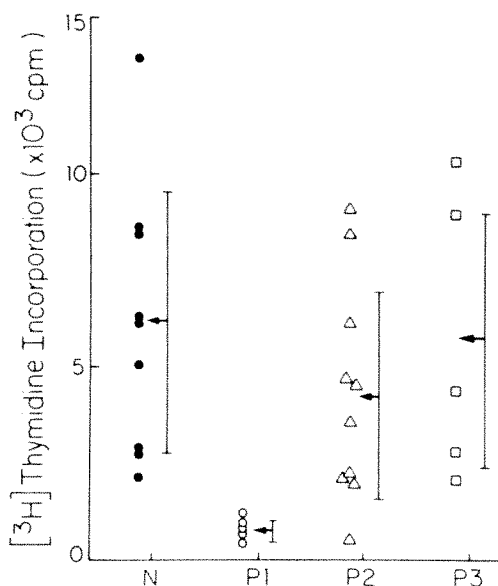


Fig 5. B cell proliferative responses to SAC in normal subjects and three groups of marrow recipients. N (●) represents the normal subjects, P1 (○) represents the short-term recipients, P2 (△) represents the long-term recipients with chronic GVHD, and P3 (□) represents the healthy long-term recipients. One hundred thousand B cells were cultured with SAC (0.005%) for three days and were pulsed with [³H]-thymidine for four hours before harvest. Each point represents the mean value of triplicate cultures. Mean values for each group were indicated with (—) and vertical bars represent one SD above and below the means.

for three days, and their proliferative responses were measured by [³H]-thymidine incorporation. Figure 5 shows that B cell proliferative responses to SAC are impaired in short-term recipients but not in long-term recipients with or without chronic GVHD.

B cell proliferative responses to T-sup after SAC activation in normals and marrow recipients. The proliferative responses of SAC-stimulated B cells from nine normal control subjects and from 20 marrow recipients were examined (Table 2). The T-sup (obtained from normal subjects) consistently maintained the proliferative responses of normal B cells after SAC activation, whereas SAC prestimulated B cells from all short-term recipients failed to proliferate in response to T-sup. One (UPN 2534) of ten long-term recipients with chronic GVHD had B cells that could not respond to any stimulation. Two patients (UPN 2416 and 1531) had B cells that responded to SAC normally but failed to maintain proliferative responses to T-sup after SAC stimulation. Seven of ten long-term recipients with chronic GVHD and all five healthy long-term recipients had B cells capable of responding to T-sup after stimulation with SAC.

B cell differentiative responses to T-sup in normals and patients. Recipient's B cells showed variable responses to SAC and T-sup co-stimulation, whereas normal B cells consistently resulted in increased Ig production in response to SAC and T-sup co-stimulation (median 14.6 µg/mL, range: 7.1 to 42.8 µg/mL) as shown in Fig 6. B cells from marrow recipients did not produce Ig spontaneously or respond to either SAC or T-sup alone (data were not shown). IgG and IgM were the predominant immunoglobulin classes produced, while lesser amounts of IgA were produced by B cells from normal subjects and marrow recipients.

Control B cells from normal subjects stimulated with SAC and T-sup together produced large amounts of immunoglobulins. In contrast, B cells from short-term recipients produced small amounts of immunoglobulin in response to co-stimulation with SAC and T-sup. Six of ten long-term recipients with chronic GVHD had B cells with poor Ig production responses to co-stimulation with SAC and T-sup. Three of these recipients had B cells that could not proliferate after either SAC or SAC plus T-sup co-stimulation, and the other three recipients had B cells that could proliferate but could not differentiate in response to SAC plus T-sup co-stimulation. B cells from the other four long-term recipients with chronic GVHD showed normal differentiative responses. Two of five healthy long-term recipients had B cells that failed to produce Ig in the range defined by normal B cells, although their B cells were able to proliferate after SAC and T-sup co-stimulation. B cells from the other three recipients without chronic GVHD showed normal differentiation responses.

Types of distribution of abnormalities. Table 3 summarizes the types and the distribution of various B cell abnormalities observed after marrow grafting. B cells from all three groups of patients showed B cell defects at one or more steps in the maturational scheme. These abnormalities include defective responses to SAC activation and failure to proliferate and/or differentiate in response to T-sup. There was no correlation between the magnitude of proliferative

Table 2. B Cell Proliferative Responses in Marrow Recipients

	[³ H]-Thymidine Incorporation (cpm × 10 ³)				
	None*	SAC (day 3)*	SAC (day 6)†	T-sup‡	SAC + T-sup§
Normals (N = 9)					
Mean	0.75	6.20	1.05	3.94	7.68
Median	0.81	6.10	1.01	4.25	7.42
Range	0.36–1.18	2.22–13.77	0.33–1.90	1.62–5.60	3.17–10.83
Short-term recipients					
UPN 2829	1.25	1.21	0.75	1.56	1.47
UPN 2909	0.29	0.86	0.28	4.72	1.92
UPN 2910	0.32	0.40	0.51	1.56	.87
UPN 2948	0.51	0.62	0.54	1.67	1.73
UPN 2936	0.29	0.75	0.52	1.26	.69
Median	0.32	0.75	0.52	1.56	1.47
Long-term recipients with chronic GVHD					
UPN 2461	0.14	9.04	0.37	5.83	8.70
UPN 2533	0.88	5.88	0.55	4.71	9.79
UPN 2416	0.41	4.56	0.27	1.25	1.06
UPN 2474	0.22	3.50	0.27	1.92	6.15
UPN 1690	0.27	2.20	0.27	1.69	4.38
UPN 2534	0.06	.51	0.16	.91	0.51
UPN 1531	0.25	4.54	0.10	1.04	0.74
UPN 2569	0.66	2.01	0.91	1.28	4.02
UPN 2503	0.34	8.41	0.72	0.99	6.12
UPN 2317	0.31	2.04	0.46	0.25	2.69
Median	0.29	4.02	0.32	1.27	4.20
Long-term recipients without chronic GVHD					
UPN 2590	0.28	2.33	0.29	3.76	6.43
UPN 2504	0.36	2.74	0.94	4.63	12.01
UPN 2525	0.19	4.45	0.38	1.28	3.33
UPN 1311	1.24	8.95	1.44	3.42	7.83
UPN 2599	0.34	10.23	0.89	1.10	26.36
Median	0.34	4.45	0.89	3.42	7.83

Purified B cells (1.0×10^5) were cultured in combinations indicated.

*Cultures harvested after three days.

†Cultures harvested after six days.

‡Cultures stimulated with T-sup alone for three days.

§Cultures activated with SAC for three days and then T-sup added for the last three days (total of six days of culture).

responses and the amount of Ig produced. However, immunoglobulin production was not observed in the absence of proliferative B cell responses. These abnormalities were found less frequently in B cells from long-term healthy recipients.

Correlation of serum immunoglobulin levels and B cell differentiation ability. Serum immunoglobulin levels in long-term marrow recipients were compared with in vitro immunoglobulin biosynthesis responses of B cells after SAC and T-sup stimulation. In vitro B cell differentiation responses to co-stimulation with SAC and T-sup were significantly correlated with the presence or absence of low serum IgG or IgM levels but not IgA levels (Fisher's exact test $P = 0.0075$).

DISCUSSION

This study demonstrates that B cell defects occur at various levels of differentiation in marrow transplant recipients. These defects include impaired proliferative responses

to SAC antigen and the inability to proliferate and/or differentiate in response to T-sup containing BCGF and BCDF activities. Such B cell defects in marrow recipients have not been reported in the literature.

Previous studies in our laboratory revealed intrinsic B cell defects in marrow graft recipients using polyclonal activators^{21–26}; however, the intrinsic defects of B cells have not been further defined. The proliferation and differentiation of B cells in response to pokeweed mitogen and other polyclonal activators is totally or partially dependent on the accessory cell function of monocytes and the presence of helper T cells.³⁴ For these reasons it has been difficult to identify the specific stage(s) of B cell defects in the maturation processes.

T cell-dependent signals for B cell proliferation and differentiation can be completely replaced by soluble factors such as BCGF and BCDF, thereby permitting the dissection of B cell maturation processes in the absence of accessory cells. Since the BCGF, BCDF, PHA, and IL 2 in crude T cell

Fig 6. B cell differentiation responses to T-sup after SAC activation in normal subjects and three groups of marrow recipients. N (●) represents the normal subjects, P1 (○) represents the short-term recipients, P2 (Δ) represents the long-term recipients with chronic GVHD, P3 (□) represents the long-term recipients without chronic GVHD. Median values for each group are indicated with (—). One hundred thousand B cells were cultured with SAC (0.005 % vol/vol) and T-sup (3.125%) for ten days. Supernatants from triplicate cultures were pooled and Ig production was quantitated by ELISA. Experimental values below the dashed lines ($\leq 20\%$ of normal median values for IgG, IgM, IgA and total Ig) were considered defective.

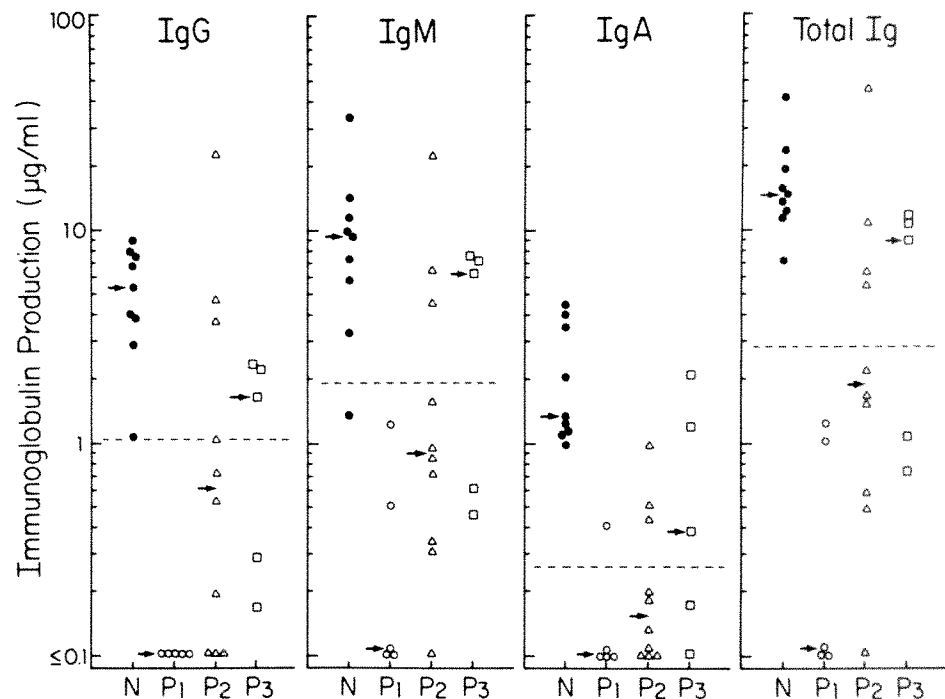


Table 3. Summary of B Cell Responses to SAC and T-sup in Marrow Graft Recipients

Patient Groups	Response		
	SAC	Proliferation	Differentiation
Short-term recipients			
UPN 2879	—	—	—
UPN 2909	—	—	—
UPN 2910	—	—	—
UPN 2948	—	—	—
UPN 2936	—	—	—
Defect/total (%)	5/5 (100)	5/5 (100)	5/5 (100)
Long-term recipients with chronic GVHD			
UPN 2461	+	+	—
UPN 2533	+	+	+
UPN 2416	+	—	—
UPN 2474	+	+	—
UPN 1690	+	+	+
UPN 2534	—	—	—
UPN 1531	+	—	—
UPN 2569	+	+	+
UPN 2503	+	+	+
UPN 2317	+	+	—
Defect/total (%)	1/10 (10)	3/10 (30)	6/10 (60)
Long-term recipients without chronic GVHD			
UPN 2590	+	+	+
UPN 2504	+	+	+
UPN 2525	+	+	—
UPN 1311	+	+	+
UPN 2599	+	+	—
Defect/total (%)	0/5 (0)	0/5 (0)	2/5 (40)

“+” denotes a normal response and “—” denotes a deficient response. Deficient response is defined by less than 30% of median value of normal in proliferative response and less than 20% of median value of normal in total Ig production.

supernatants may cause T cell proliferation, and monocytes in the non-T cell fractions may inhibit the B cell responses to T cell factors.^{35,36} Vigorous depletion of T cells and monocytes from B cell-enriched fractions was necessary. Our results show that B cell fractions had nearly background responses to PHA stimulation. However, we could not exclude the possibility that the soluble factor(s) such as IL 2 in the T-sup stimulated small numbers of contaminating T cells to proliferate or secrete other factor(s) that in turn may modulate B cell proliferation or differentiation. Howard et al³⁷ demonstrated that IL 2-stimulated T cells secrete BCGF. Recent studies demonstrated that activated B cells express IL 2 receptors and can proliferate and differentiate in response to recombinant IL 2.⁵⁻⁷ These data support the notion that IL 2 is acting as a growth and differentiation factor for B cells. Since IL 2 was not absorbed from T-sup containing BCGF and BCDF activity and highly purified preparations of BCGF and/or BCDF were not available, our results likely represent B cell reactivity to BCGF, BCDF, and also to IL 2. The effect of recombinant IL 2 on the growth and differentiation of B cells from marrow recipients is under investigation.

B cells from all short-term recipients and one long-term recipient with chronic GVHD failed to show appreciable responses to any stimulation. The cause of this type of B cell defect is unknown, but it is possible that these B cells could not bind SAC or transmit the SAC activation signals. These B cells would presumably fail to express receptors necessary for proliferation and differentiation due to a defect in SAC activation. We hypothesize that the defect in SAC activation is the result of a maturational arrest that occurs early in B cell ontogeny. Ault et al³⁸ described an unusual B cell observed shortly after transplant that expressed B markers (sIg+ and B1) and the CD5 antigen but no other T cell antigens. This B cell bearing the CD5 antigen was also found

in fetal spleen and did not respond to pokeweed mitogen stimulation by producing Ig.³⁹ The lack of in vitro function and time of appearance after marrow grafting suggest that these CD5 positive B cells may be associated with the unresponsiveness of recipient B cells to SAC activation observed after marrow grafting.

In some long-term recipients, B cells were unable to proliferate and/or differentiate in response to T cell supernatants even though they showed initial proliferative responses to SAC. Although the causes of such defects are unknown, it is reasonable to postulate that such B cell defects may be due to the failure to express receptors for proliferation and/or differentiation factors after SAC activation or due to post-receptor abnormalities within the B cells. Another possibility is that low-level monocyte contamination may account for deficient B cell proliferation and differentiation. In a previous study²⁶ we found that some recipient monocytes could suppress Ig production from normal B cells in the pokeweed mitogen system. These results are consistent with our previous studies in that B cells from nearly all short-term patients, one-half of the long-term recipients with chronic GVHD, and roughly one-third of the long-term recipients without chronic GVHD could not differentiate into Ig-secreting cells in the presence of normal helper T cells in the pokeweed mitogen-stimulated cultures.²¹⁻²⁶

Although the B cell defects delineated herein have not been previously described, similar findings were observed in patients with common variable immunodeficiency (CVI). Saiki et al⁴⁰ reported that a group of CVI patients showed normal B cell proliferation to SAC but failed to differentiate in the presence of T cell factor. However, they did not evaluate B cell proliferation to T cell factor after SAC activation. Recently Perri and Weisdorf⁴¹ reported a case of a CVI patient with a defective response to BCGF. Such defects in B cells from marrow recipients and CVI patients may contribute to the impaired in vivo humoral antibody responses.

On the other hand, Saxon et al⁴² reported three cases of chronic GVHD patients with hypergammaglobulinemia who had B cells that spontaneously synthesized Ig in vitro. They postulated that activation and terminal differentiation of B cells occurred during chronic GVHD. In a separate series of experiments we also observed a similar case of a marrow recipient with chronic GVHD who showed hypergammaglobulinemia and increased spontaneous IgG production in vitro (unpublished observation).

In previous studies using the pokeweed mitogen and tetanus toxoid systems, we could not show any correlation between in vivo and in vitro antibody production. This study shows a significant correlation exists between in vivo serum Ig levels and in vitro differentiation responses of B cells in long-term recipients. These findings suggest not only that the assay systems used in this study more accurately reflect in vivo B cell function than PWM or tetanus toxoid-stimulated systems but also that B cell differentiation defects are a major cause of defective antibody responses seen in vivo after marrow transplantation.

Taken together our present results and other studies show that B cell deficiencies occur at various levels of differentiation following bone marrow transplantation. Further studies using monoclonal antibodies directed at the receptor(s) for BCGF and BCDF and the use of recombinant preparations of various B cell active lymphokines may provide important insights into these abnormalities. The results of such studies may eventually permit the use of recombinant forms of BCGF and/or BCDF for therapeutic intervention in marrow transplant recipients and immunodeficiency disorders.

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Ektacytometric Measurement of Sickle Cell Deformability as a Continuous Function of Oxygen Tension

By Martin P. Sorette, Marc G. Lavenant, and Margaret R. Clark

In an effort to study the rheologic effects of small amounts of hemoglobin S (HbS) polymer in sickle red cells, we have used the ektacytometer, a laser diffraction couette viscometer, to measure sickle cell deformability as a function of oxygen tension. Sick cell populations of defined intracellular hemoglobin concentration (MCHC) were isolated using Stractan density gradients and were resuspended in buffered polyvinylpyrrolidone solutions for deformability measurements. Using a gas-porous, hollow fiber gas exchange system to establish a linear gradient in oxygen tension, deformability was measured over a pO_2 range of 76 to 0 mm Hg. Parallel spectroscopic determinations of oxygen saturation permitted determination of cell de-

mability as a function of oxygen saturation for each discrete MCHC population. From these measurements the level of oxygen saturation at which a loss in cell deformability was first detected could be defined. Then, using the data of Noguchi and Schechter,⁹ the amount of polymerized HbS in the cells at that defined level of oxygen saturation was estimated. The results of this analysis suggested that the quantity of polymer that caused a detectable loss in cell deformability increased with increasing MCHC. In addition, for MCHC above 30 g/dL, this represented a substantial fraction of the total HbS in the cell.

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IT IS WIDELY ACCEPTED that the pathologic manifestations of sickle cell disease arise from abnormalities in blood rheology that ultimately derive from the formation of rigid hemoglobin S (HbS) polymer within the cells. Several different factors that influence blood rheology could potentially contribute to clinical problems: acute loss of deformability in red cells that develop polymer at low oxygen tension¹⁻³; chronically impaired deformability of red cells that have been dehydrated, possibly as a result of previous polymerization episodes⁴; impaired capillary blood flow because of increased adherence of sickle red cells to the endothelial lining of the vessels⁵; as well as factors other than sickle red cells that modulate the function of the microvasculature.⁶ Recently nuclear magnetic resonance (NMR) studies have provided evidence that sickle cells from the upper end of the distribution in mean cell hemoglobin concentration (MCHC) maintain polymeric HbS even at relatively high levels of oxygen saturation.⁷⁻⁹ On this basis it has been suggested that the continuous presence of polymer in circulating sickle cells may provide a dual hazard. First, pre-formed polymer would eliminate any delay in formation of more polymer in the event of a rapid transient reduction in oxygen tension; and second, the residual polymer might itself impair the deformability of the red cell, reducing its ability to circulate through the microvasculature.

In an effort to evaluate the physiologic significance of this latter possibility, we have employed a means of measuring sickle cell deformability at low oxygen tension similar to those used by Johnson et al.,¹⁰ Bessis et al.,¹¹ and Feo et al.¹² By

measuring the loss of cell deformability as a continuous function of oxygen tension, we have sought to determine the effect of a relatively defined intracellular concentration of HbS polymer on red cell rheology.

MATERIALS AND METHODS

After obtaining informed consent, as approved by the Human and Environmental Protection Committee of the University of California, San Francisco, blood samples were drawn into acid-citrate-dextrose (ACD) or EDTA. Patients were identified as being homozygous for HbS on the basis of hemoglobin electrophoresis and family studies performed by the Northern California Comprehensive Sickle Cell Center. To measure red cell deformability we used the ektacytometer, a laser diffraction couette viscometer that has previously been described in detail.¹³ For the present experiments the instrument was coupled to a gas exchange system similar to that described by Schmuklar and Chien,¹⁴ which permitted the preparation of cell suspensions at defined oxygen tension (Fig 1). The gas exchange system was a bundle of 200 μ m diameter gas-porous, hollow fibers (Celgard type X-10 MHF; Celanese Corp, Charlotte, NC), around which flowed a humidified mixture of nitrogen and oxygen at the desired oxygen tension. The fibers were sealed into an airtight plexiglass housing with gas-tight compression fittings at the entry and exit ports. The red cells, suspended in a solution of 4% (wt/vol) polyvinylpyrrolidone (PVP) in isotonic buffered salt solution, were pumped through the fibers by a Harvard infusion pump (Model 600-000, Harvard Apparatus Co, Dover, MA) at a rate of 3.8 mL/minute. The suspension then flowed into a length of oxygen-impermeable tubing (Nyliflow pressure tubing 1/8 in, Polymer Corp, Reading, PA) immersed in a water bath at 37 °C, where it was held for varying periods to permit polymer formation. In normal operation a proportional metering valve was used to vary the proportion of oxygen to nitrogen in the gas exchange mixture over a chosen range as the cell suspension flowed through the gas exchange system. The fibers in which gas exchange occurred contained a cell suspension volume of 2.7 mL, and the full gradient of 76 mm Hg was established over a cell suspension volume of 30 mL. Once the oxygen gradient was established along the flowing cell suspension, the cells passed into a length of small diameter, gas-impermeable tubing, where they were equilibrated at 37 °C to permit polymer formation. The oxygen gradient was found to be maintained along the tube length for periods up to 90 minutes.

After equilibration the cell suspension was pumped past an oxygen electrode (Model E 5046; Radiometer, Copenhagen) fitted with a high sensitivity rapid response membrane (Yellow Springs Instru-

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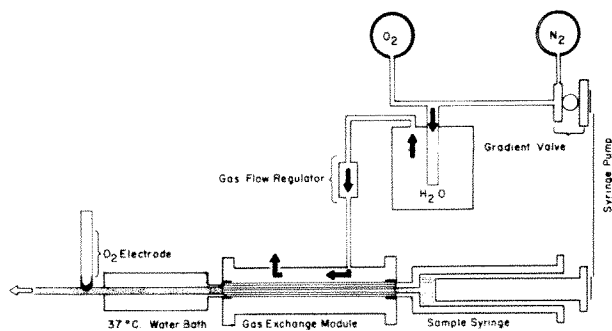


Fig 1. Schematic diagram of the sickle cell deoxygenation system. As described in Materials and Methods, a bundle of gas-porous, hollow fibers was used to prepare cell suspensions whose oxygen tension varied from 76 to 0 mm Hg along the length of tubing in which they were contained.

ment Co, Yellow Springs, OH) and then into the bottom of the ektacytometer chamber. Aspiration of the flowing suspension from the top resulted in an upward flow through the annular space between a stationary and a rotating cylinder. A viscometer rotation speed of 100 rpm generated a laminar shear stress field of approximately 100 dynes/cm² for 4% PVP concentration. This level of shear stress, at the upper range of that expected *in vivo*,¹⁵ was selected because it would induce deformation of nearly all normal red cells, whereas high MCHC normal and sickle cells would not deform at lower applied shear stress. It should be noted that deformation under these conditions does not imply that the cells deform readily *in vivo*. A laser beam passing through the cell suspension was diffracted by the cells, and a photometric detector provided a deformability index (DI) signal. This signal can be decreased in either of two instances.¹³ First, a subpopulation of cells could become undeformable, and a double diffraction pattern representing both deformable and undeformable cells would be seen. Alternatively, the entire cell population might show a homogeneous reduction in the extent of deformation, producing a single diffraction pattern. In these experiments a double diffraction pattern was observed over the range of decrease in the DI signal, indicating that cells either deformed fully or failed to deform. Therefore, the DI signal gave a measure of the proportion of deformable cells that persisted at any given pO_2 .

For each deformability measurement, approximately 250 μ L of packed cells were suspended in 50 mL of isotonic PVP-buffer solution (0.5% hematocrit). The cation composition of the buffer was chosen to match the average intracellular concentrations (in mEq/L cell water) of the sickle cell samples. The purpose of this was to minimize the transmembrane gradients for Na and K, thereby preventing changes in intracellular ion and water content once the cells sickled and became more permeable to cations. On the basis of flame photometric measurement (Instrumentation Laboratories Model 443, Lexington, MA) of the sodium and potassium content of sickle cells washed in isotonic Tris-buffered (10 mmol/L, pH 7.4) $MgCl_2$ solutions, we used a medium containing 17 mmol/L NaCl, 50 mmol/L KCl, and 10 mmol/L Na phosphate, pH 7.4 or 7.2. Sucrose was added to a concentration of 123 mmol/L to adjust the osmolality to 290 mosm/kg. The addition of PVP has a negligible effect on osmolality. In most experiments the concentration of PVP was 4 g/100 mL of buffer, but in some experiments the concentration was varied to determine the effect of suspending medium viscosity. The viscosity of the PVP solutions at 37 °C was measured using a capillary viscometer.

To relate the observed changes in cell deformability to oxygen tension, the DI signal was applied to the Y axis of an XY recorder, while the signal from the oxygen electrode was applied to the X axis.

The oxygen electrode was calibrated using flowing PVP-buffer solutions that had been equilibrated with defined gas mixtures.

In these experiments, for each set deformability measurements it was important to know the actual concentration of deoxy HbS within the cells as a function of oxygen tension. To determine this we prepared cell suspensions along the oxygen gradient exactly as for the ektacytometric measurements, but after the equilibration period pumped them through a flow-through cuvette in a Beckman DU7 spectrophotometer (Beckman Instruments, Palo Alto, CA). As the cells flowed past the oxygen electrode and through the cuvette, repeated measurements of absorbance at 540, 560, and 576 nm were made for every 3.8 mm Hg change in pO_2 . Using fully deoxygenated and fully oxygenated cell suspensions, a correction for light scattering by the cells was determined. The corrected absorbance data were then used to calculate the percentage of deoxyhemoglobin at each time point, using the equations of Benesch et al.¹⁶ Care was taken to ensure that the cell suspension volume between the oxygen electrode and the light path in the cuvette was the same as the corresponding volume in the parallel ektacytometric experiments. This obviated the need to correct for the spatial separation of the viscometer and the oxygen electrode when DI data were related to oxygen saturation. The measured values of oxygen saturation and MCHC were then used to estimate intracellular polymer content at the values of oxygen tension at which deformability changes occurred, using data from Noguchi.¹⁷ This involved taking interpolated values from the graphic representation of their calculated curves for polymer fraction as a function of oxygen saturation for cells of various MCHC over the range of 24 to 48 g/dL. To permit use of this approach, which does not take into account the presence of hemoglobins other than HbS, only samples containing low HbF (<8%) were used in this analysis.

Whole sickle blood contains cells covering a broad range in MCHC, which has a large effect on HbS polymerization. Therefore we used density gradient centrifugation to isolate more homogeneous MCHC populations in which polymer content could be more closely defined. We used arabinogalactan (Stractan II; St Regis Paper Co, Tacoma, WA)¹⁸ to prepare discontinuous gradients, using six layers ranging in equal steps from 1.085 to 1.110 g/mL density, layered onto a cushion of at least 1.133 g/mL density. The gradients were centrifuged in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 30 minutes at $74,000 \times g$ at 20 °C. The separated cells were collected from the gradient interfaces using a Pasteur pipet and were washed three times in buffered saline containing glucose and potassium (135 mmol/L NaCl, 5 mmol/L KCl, 8.6 mmol/L Na_2HPO_4 , 1.4 mmol/L NaH_2PO_4 , and 11 mmol/L glucose, adjusted to pH 7.4 and 290 mosm/kg) before use. MCHC was determined from a spun hematocrit and spectrophotometric measurement of hemoglobin as the cyanomethemoglobin complex. Fetal hemoglobin was quantitated using isoelectric focusing techniques.¹⁹ For evaluation of sickle cell morphology, a double gas exchange system was constructed so that glutaraldehyde solutions and red cell suspensions could be adjusted to identical pO_2 . The effluent from both sets of fibers was then brought together to fix the cells without altering their oxygen saturation. The percent of sickled cells was determined from counts of 500 cells.

RESULTS

When a gradient in oxygen concentration from 76 to 0 mm Hg was established along a flowing suspension of sickle cells and the cells allowed to equilibrate for 30 minutes, they showed the expected oxygen concentration-dependent reduction in the ability to deform under shear stress, as shown in Fig 2. The DI v pO_2 curves showed a constant, maximal DI

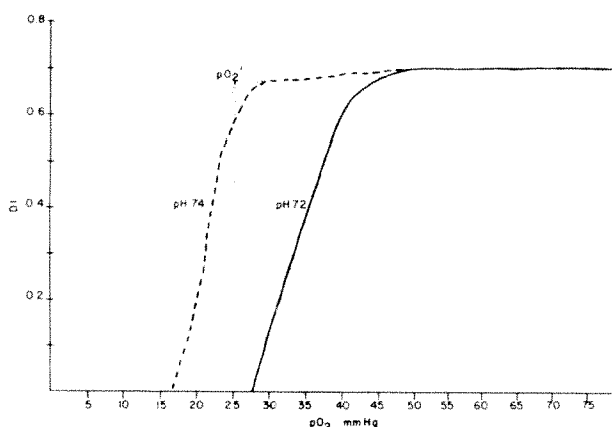


Fig 2. Variation in deformability signal (DI) as a function of oxygen tension for a Fraction 2 gradient population. As shown here, cell deformability decreased abruptly over a relatively narrow range in pO_2 , producing a curve that essentially represented two intersecting lines. The point of intersection was used to define the pO_2 at which loss of deformability was first detected (pO_2^*). Reduction of the pH from 7.4 to 7.2 shifted this critical pO_2 to higher values, reflecting the pH-induced increase in HbS polymerization.

down to a critical pO_2 level, after which it decreased in essentially linear fashion. Thus, the intersection of the two linear portions of the curve was used to identify the pO_2 value at which a loss of deformability was first detectable (designated pO_2^* in Fig 2). In addition, because this value was expected to vary with MCHC, we also used the pO_2 at which the DI fell to a value of 0.2 to characterize each curve. The data shown in this figure and in Figs 3 through 5 were obtained on cell populations collected from the interface between the first and second layers of Stractan (Fraction 2). Such density-separated cell populations displayed a sharper transition in deformability with oxygen tension than whole blood, as expected from their greater homogeneity (data not

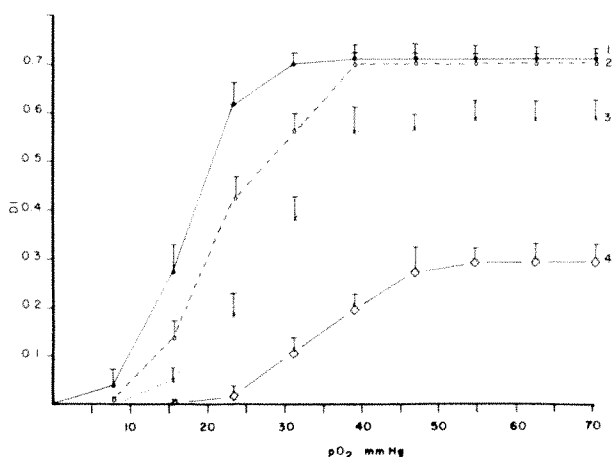


Fig 3. Effect of suspending medium viscosity on the DI v pO_2 curve for a Fraction 2 gradient population. A decrease in viscosity below 19.5 cp at 37 °C cp (4% PVP concentration) resulted in overall reduction in cell deformation and eventual blurring of the deoxygenation-dependent loss of cell deformability. (-----) 19.5 cp, (—) 12.5 cp, (—) 9.5 cp, (·····) 7.5 cp.

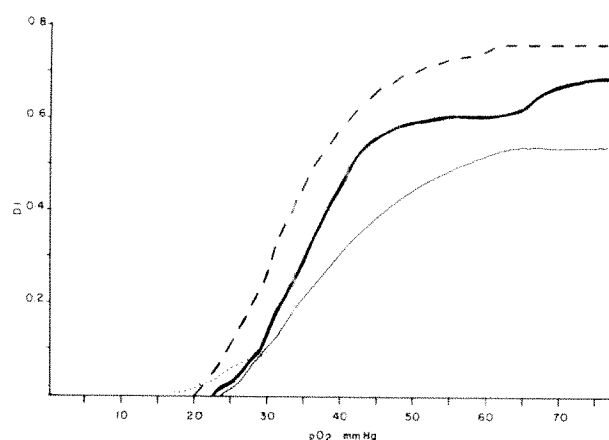


Fig 4. Comparison of DI v pO_2 relationship for continuous pO_2 gradient (continuous curve) and for measurements performed at fixed pO_2 values (open circles) over the same range for a Fraction 2 gradient population.

shown). It can be seen that these Fraction 2 samples in the various experiments did not give identical DI v pO_2 curves. Comparison of the oxygen dissociation curves for the samples showed that differences in oxygen affinity, presumably due to individual patient differences and differences in cell storage time, were the major source of variation.

A series of experiments was performed to determine the effect of important experimental variables and to define the optimal procedure to obtain reproducible, physiologically relevant data. As expected from the characteristics of the polymer equilibrium, several factors predictably affected the range of oxygen tension over which the cell deformability loss occurred. Reduction of the suspending medium pH from 7.4 to 7.2 caused a parallel shift of the DI v pO_2 curve to higher pO_2 (Fig 2), consistent with the increased Bohr effect of HbS, particularly below an extracellular pH of 7.4, and the corresponding enhancement of polymer formation.^{4,20} Most of the experiments employed paired deformability and oxygen saturation measurements at both pH 7.2 and 7.4 to permit comparison of the results with those of Noguchi et al⁷ and Noguchi¹⁷ on polymer content, since those experiments

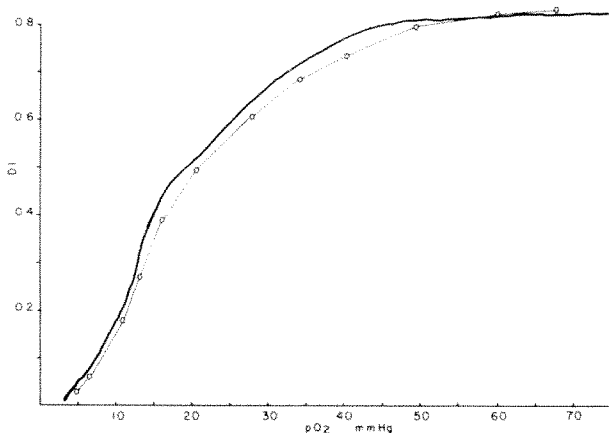


Fig 5. Relationship between the reduction in the DI signal and percentage of sickle cells for a Fraction 2 gradient population.

were performed at pH 7.2. Some additional experiments were performed only at pH 7.4.

The position of the DI v pO_2 curve also varied with MCHC, as shown by measurements using subpopulations of sickle cells isolated on density gradients (Fig 6). Again, the shift of the curves to higher pO_2 with increasing MCHC was consistent with the known increase in polymer content with increasing MCHC for any given oxygen tension.^{4,21} The DI versus pO_2 curves for unseparated cells (data not shown) were always broader than those for the gradient populations, reflecting the fact that the heterogeneous mixture of cells underwent polymerization and sickling over a broad pO_2 range.

Another variable that influenced the position of the DI v pO_2 curve was the viscosity of the suspending medium. A decrease in extracellular viscosity, achieved by reducing the concentration of PVP, resulted in a reduction in the proportion of deformable cells at progressively higher pO_2 values. For higher MCHC cells there was also a reduction in the maximum attainable DI (Fig 3). We attribute this latter effect to the requirement for a threshold ratio of extracellular to intracellular viscosity to induce red cell deformation.²² A viscosity of 19.5 cp (4% PVP at 37 °C) was chosen for subsequent experiments, since this viscosity showed clear-cut deformability differences among normal red cells from different density gradient subpopulations without introducing large variations among whole blood samples from different normal subjects. It was found that if the viscosity was reduced much below 19 cp, a substantial and varying proportion of cells from normal individuals would not deform, leading to a broadening of the range of normal isotonic DI values.

Other experimental variables tested for their effect on the deformability-oxygen tension relationship included the cation composition of the suspending medium, the flow rate of the cell suspension through the gas exchange fibers, and the time period between gas exchange and deformability measurement. When cells were equilibrated in high-Na,

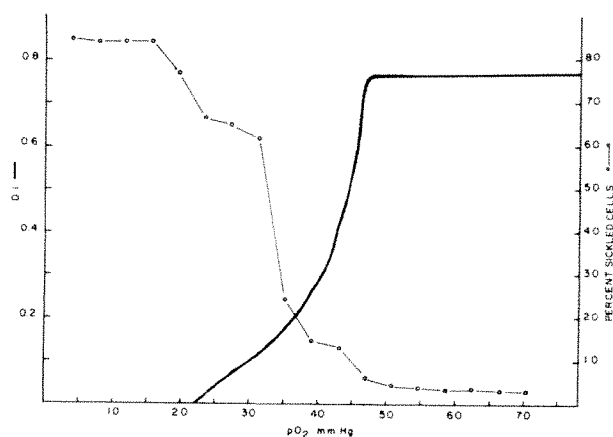


Fig 6. DI v pO_2 curves for Fractions 1 to 4 of Stractan-separated sickle cells. As the MCHC of isolated cell populations increased, the maximum DI decreased, and a deoxygenation-dependent loss in cell deformability occurred at progressively higher pO_2 . See Table I for characteristics of the cell populations.

Table 1. Characteristics of Sickle Cell Gradient Subpopulations

Fraction	MCHC (g/dL)	Reticulocytes (%) ($\bar{X} \pm \text{SEM}$)	ISC (%)
1	26.00 $\pm$ 0.72	10.95 $\pm$ 1.14	2.12 $\pm$ 0.29
2	29.49 $\pm$ 0.44	7.84 $\pm$ 1.09	2.95 $\pm$ 0.48
3	31.21 $\pm$ 0.47	5.59 $\pm$ 0.55	3.82 $\pm$ 0.46
4	33.20 $\pm$ 0.47	4.22 $\pm$ 0.55	5.95 $\pm$ 0.60
5	34.75 $\pm$ 0.37	2.83 $\pm$ 0.80	7.58 $\pm$ 0.93
6	39.48 $\pm$ 0.91	1.40 $\pm$ 0.19	12.59 $\pm$ 1.08

low-K medium, the DI- pO_2 curve was shifted to higher oxygen tensions as compared to the curve obtained for cells from the same sample that were equilibrated in a medium in which the Na and K concentrations were close to their intracellular concentrations. This difference was interpreted as a reflection of a net leakage of K ions out of sickled cells in the high Na medium, resulting in a loss in cell water and a concomitant increase in HbS concentration and polymer content. Therefore the buffer that minimized transmembrane cation gradients was used for the rest of the experiments.

The rate at which the cells were pumped through the gas exchange fibers affected the subsequent deformability of even fully oxygenated cells. If the flow rate was high (>4 mL/min), the maximum cell deformation was decreased. We surmise that this was likely the result of shear-induced fragmentation occurring as the cells entered or left the fibers. Calculation of Reynolds numbers for flow through the fibers at these rates indicates that turbulence was probably present, increasing the likelihood of cellular fragmentation. The delay time for polymer formation at low HbS concentration and at pH 7.4 was expected to be prolonged, particularly at higher levels of oxygen saturation.²³ Therefore, we wanted to determine the maximum period for which the cells could be equilibrated at 37 °C without undergoing damage and alteration in their deformability properties that could not be explained on the basis of HbS polymer formation. Variation of the equilibration period from 30 to 90 minutes had no significant effect on the DI v pO_2 curve (data not shown). An equilibration period of 30 minutes was chosen for the study.

We performed experiments in which 10-mL aliquots of cell suspension were run through the system while a constant pO_2 was maintained in the gas exchange system (Fig 4). Measurement of the DI at various fixed pO_2 values along the gradient range showed that the DI- pO_2 relationship obtained was very close to the continuous curve obtained using the decreasing oxygen gradient (when the continuous curve was adjusted for the spatial separation of the oxygen electrode and the ektacytometer). Therefore, we conclude that measurements performed using a decreasing gradient from 76 to 0 mm Hg oxygen provide accurate information about the proportion of sickle cells that can deform after equilibration at a given pO_2 value.

Examination of cell morphology at various points along the pO_2 gradient allowed us to define the relationship between the reduction in the DI signal and the percentage of sickle cells in a representative isolated cell population with an MCHC of 32 g/dL at pH 7.2 (Fig 5). We observed both characteristic elongated cells with spicules, and a larger

number of crumpled, or "holly leaf," cell forms, all of which were counted as sickled cells. We note that sickle forms appear in the fixed cell population prior to a point where there is a decrease in the DI signal, and that some discoid cells persist even at very low pO_2 .

Using the conditions defined as described above, we obtained DI v pO_2 curves for 34 samples of density-separated sickle cells from 14 individuals, covering an MCHC range from 27 to 34 g/dL. These curves were characterized by two parameters: the pO_2 at which the deformability loss was first detectable, and the pO_2 at which the DI signal fell to 0.2 (Fig 2). Then the parallel oxygen saturation measurements were used to determine the percent oxygen saturation at these pO_2 values. These data, summarized in Fig 7, show that both the pO_2 and oxygen saturation at which polymer formation interfered with cell deformability rose with increasing MCHC.

To relate the observed deoxygenation-dependent changes in sickle cell deformability to HbS polymer concentration, we used the estimates of Noguchi and her associates for polymer content at a given pO_2 and intracellular HbS concentration.^{7,17} Using data only from samples containing less than 8% HbF, we have plotted the estimated polymer content resulting in the first detectable decrease in cell deformability as a function of MCHC (Fig 8). Since Noguchi's estimates were based on a suspending medium pH of 7.2 and since we were interested in the more physiologic pH of 7.4, we determined the DI as a function of oxygen saturation at both pH 7.2 and 7.4 and made the assumption that equivalent decreases in DI represented equivalent increases in polymer concentration at the two pH values. When the characteristic pO_2 values were thus used to estimate polymer content at which a loss of deformability could first be detected, two observations emerged. First, the minimal detectable polymer increased with increasing MCHC. Second, in the higher MCHC cells it constituted a substantial fraction of the total hemoglobin inside the cells. Thus while it was 5% of total hemoglobin at 27 g/dL, polymer represented 35% at 34 g/dL at pH 7.2.

DISCUSSION

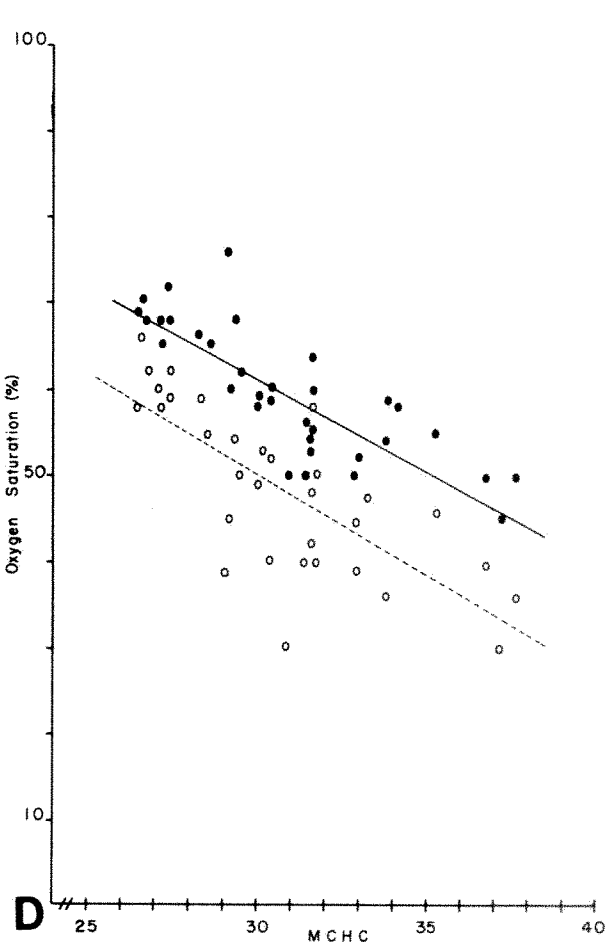
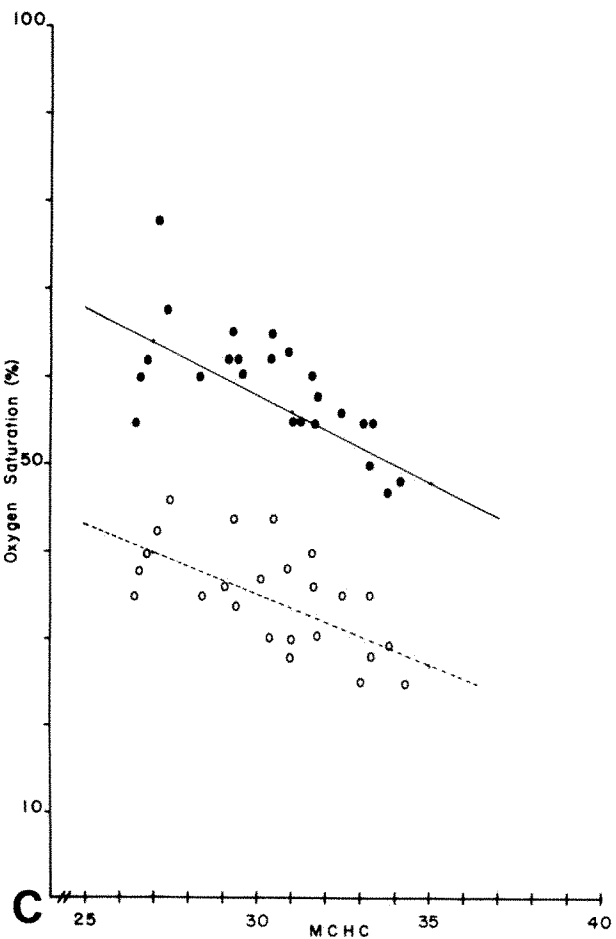
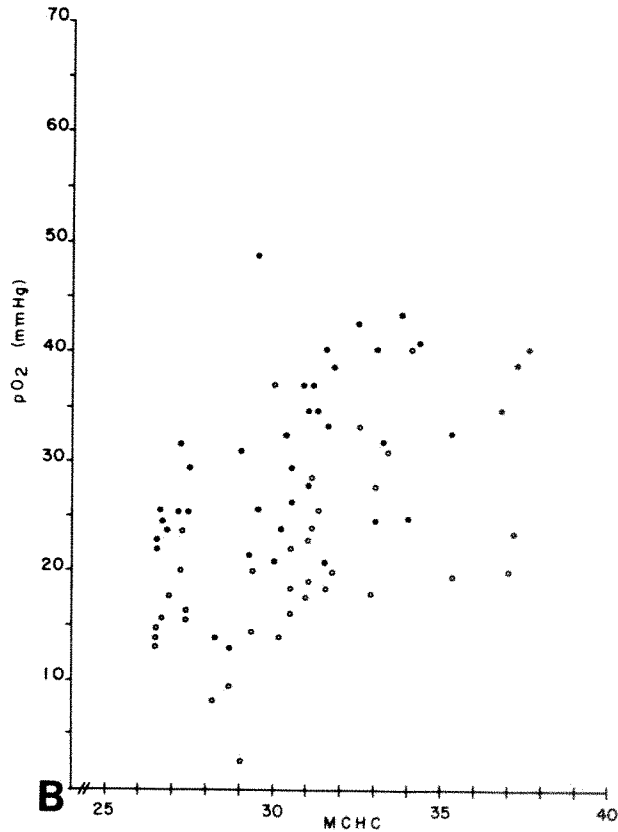
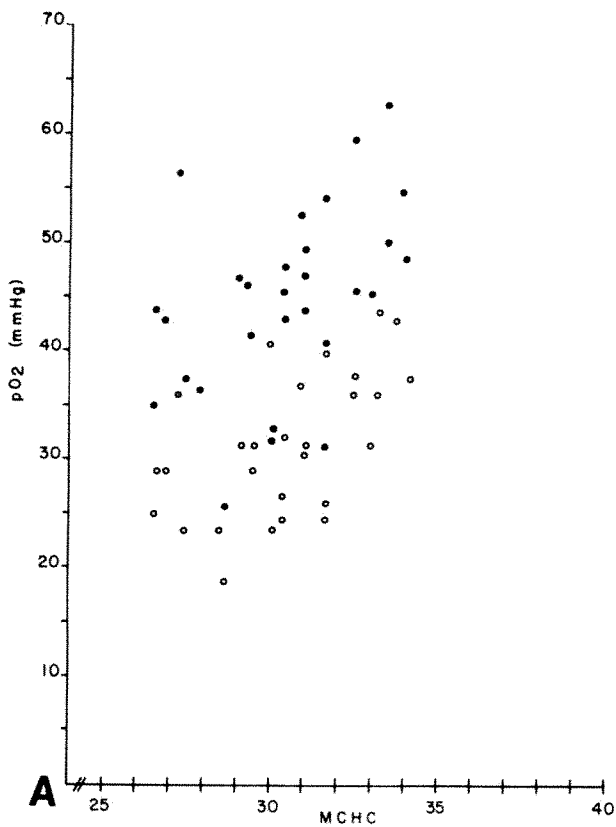
The present study provides new information concerning the rheologic effect of HbS polymer within red cells. While the physiologic interpretation of the results is limited by the fact that the threshold for loss of cell deformability depends on the selected experimental conditions, comparison with the rheologic effect of variations in MCHC and intracellular viscosity¹³ provides some perspective on the problem. The selected conditions for these experiments clearly distinguish oxygenated higher MCHC sickle cells from normal cells in their rheologic behavior, while oxygenated low MCHC cells are not distinct in their behavior. (This essentially repeats our previous observations for fully oxygenated, density-separated sickle cell populations.^{13,24}) Moreover, deoxygenation results in a reduction in the ability of low MCHC sickle cells to deform and in an additional loss in the already reduced deformability of higher MCHC cells. The diffraction pattern generated by the ektacytometer at reduced oxygen tension indicates the presence of a double population

of cells, deforming and nondeforming. Previously, it has been shown that the stably oriented, ellipsoidal pattern characteristic of red cells deforming under laminar shear stress requires a "tank-treading" motion of the membrane around the cell interior.²⁵ Thus, the loss of cell deformability in the ektacytometer can be interpreted as a loss in the ability of the cell to undergo this dynamic, tank-treading motion. This is different from the ability of a cell to undergo gradual deformation from one stationary geometric form to another.

From the data presented in our cell morphology experiment, we have determined that the percent reduction in DI signal for a given percentage of sickle cells is greater than might be predicted from the linear decrease found with glutaraldehyde-fixed normal cells.¹³ There are at least two possible explanations for this result. First, reversibly sickled cells may orient in the shear field of the ektacytometer in a direction perpendicular to that of deforming cells, much like irreversibly sickled cells (ISC). Bessis and Mohandas²⁵ have demonstrated that ISC produce a characteristic horizontal diffraction pattern that results from the perpendicular orientation of these cells to the direction of fluid flow, thus subtracting from the DI signal. A second possibility is that those cells that maintain a discoid appearance may contain sufficient HbS polymer to be partially or totally undeformable. However, recent studies suggest that the latter possibility is unlikely to contribute in a significant way to the interpretation of these data. Nash et al²⁶ and Chien et al²⁷ have noted that alterations in the viscoelastic properties of sickle cells were detected only in those cells that had undergone a morphologic change. Additional kinetic studies by Mozzarelli et al²⁸ were also consistent with these findings. Those authors, using a measurement of the delay time for HbS polymerization to detect preexisting polymer, found that whenever polymer was present, the cells were morphologically sickled.

Taking this into consideration, we suggest that the reduced deformation of sickle cells at low oxygen tension in the ektacytometer reflects the accumulation of an internal mass of polymerized HbS to a size sufficient to interfere with the tank-treading motion of the cell. It should be noted that in deoxygenation experiments such as those conducted here, polymer formation at higher oxygen tensions does not reach equilibrium during the period of experimental observation.²⁸ At these intermediate oxygen tensions, the hemoglobin solution is effectively supersaturated. This probably explains

Fig 7. Relationship between MCHC and critical pO_2 or oxygen saturation values at which loss of cell deformability was first detected (closed circle) and at which the DI fell to a value of 0.2 (open circle). (A) MCHC v pO_2 at pH 7.2. Linear regression analysis gave a correlation coefficient of $r = 0.661$ for initial loss of deformability and $r = 0.669$ for DI equal to 0.2. (B) MCHC v pO_2 at pH 7.4. Linear regression analysis gave a correlation coefficient of $r = 0.635$ for initial loss of deformability and $r = 0.571$ for DI equal to 0.2. (C) MCHC v O_2 saturation at pH 7.2. Linear regression analysis gave a correlation coefficient of $r = 0.663$ for initial loss of deformability and $r = 0.575$ for DI equal to 0.2. (D) MCHC v O_2 saturation at pH 7.4. Linear regression analysis gave a correlation coefficient of $r = 0.812$ for initial loss of deformability and $r = 0.761$ for DI equal to 0.2.



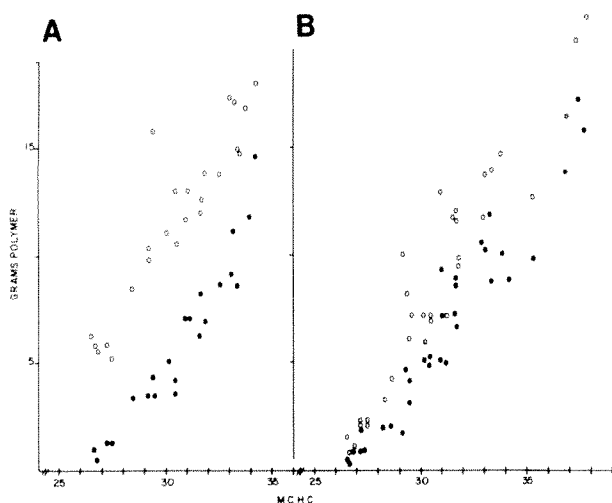


Fig 8. Estimated polymer concentration producing a loss in cell deformability for cells of various MCHC. (A) pH 7.2 (B) pH 7.4. The closed circle symbols designate the first detectable loss of deformability, and the open circle symbols designate a decrease in average DI to 0.2. The polymer fraction was estimated using the data from Noguchi¹⁷ as described in Methods.

why the DI signal in our experiments fell sharply over a narrow pO_2 range.

Under the conditions of our studies of low and medium density sickle cells, a decrease in sickle cell deformability could be detected only when the oxygen saturation was reduced to a level at which substantial concentrations of polymer are expected to be formed. This finding is consistent with the recent micropipette studies that demonstrated that enough polymer must form within a cell to cause morphologic change before deformability is impaired.^{26,27} It might have been anticipated that micropipet measurements of membrane deformation at the periphery of the cell would be less sensitive than the ektacytometer to the presence of polymer in the cytoplasm, but such a difference is not apparent in these results. Thus, small amounts of polymer may not interfere greatly either with the tank-treading or viscoelastic deformation of the cells. In addition, our results suggest that the minimum amount of polymer that interferes with cell deformation increases with increasing intracellular

Hb concentration. This could arise because increasing intracellular viscosity obscures the detection of the initial effects of polymer. Alternatively, it may be that HbS polymerization at low HbS concentration results in gradual growth of an extended network of polymer originating from a few nucleation sites, whereas polymerization at high HbS concentrations results in a rapid, multicenter growth of smaller domains of polymer.²⁹ It might be anticipated that the effect of extended polymer structures would be more effective in preventing cell deformation than a larger quantity of small, independent aggregates.

Whatever the reason for the difference in the minimum detectable quantity of polymer in cells of different MCHC, it is of particular interest that the higher MCHC cells appear less sensitive to polymer formation than the low MCHC cells. It has been proposed that the small amounts of polymer that appear to persist in high MCHC cells should have two effects of potential clinical significance.⁹ First, the presence of polymer would eliminate any delay in formation of polymer during brief periods of reduced oxygen tension. Second, the polymer might impair the cells rheologically so that they would be less able to flow rapidly through hazardous areas of low oxygen tension. Although their high intracellular viscosity prevented us from studying this process in very high MCHC cells, our results suggest that small amounts of persistent polymer would have the least rheologic effect on the cells in which it is most likely to be found. Thus, we would anticipate that any deleterious effect of persistent polymer in high MCHC sickle cells at higher pO_2 would be more likely to be exerted through its effect on the rate of polymer formation than on the deformability of cells at high levels of oxygen saturation.

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Human Neutrophil Degranulation During Extracorporeal Circulation

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Cardiopulmonary bypass, especially when prolonged, may result in hemostatic failure and pulmonary dysfunction, which has been attributed to changes in platelets and leukocytes, respectively. It has been well documented that contact of blood with synthetic surfaces causes platelet activation. In this report, we explore mechanisms of the activation of neutrophils during simulated in vitro extracorporeal circulation and document the release of neutrophil lactoferrin and elastase during clinical cardiopulmonary bypass (CCB). Inhibition in the simulated circuit by prostaglandin E_1 (PGE_1) and lidocaine suggests different mechanisms for release of neutrophil-specific proteins. During CCB with a bubble oxygenator it was observed that platelet counts fell to $42\% \pm 2\%$ of baseline. In addition, β -thromboglobulin antigen (β TG), a platelet-specific, α -granule protein marker reflecting the release reaction, increased from 0.15 ± 0.05 to $0.84 \pm 0.11 \mu\text{g/mL}$. Neutrophil counts decreased to $67\% \pm 7\%$ of prebypass levels but then gradually rose as bypass continued. Both lactoferrin, a

neutrophil-specific granule marker, and neutrophil elastase, an azurophilic granule marker, increased in plasma threefold to $1.66 \pm 0.33 \mu\text{g/mL}$ and $1.65 \pm 0.68 \mu\text{g/mL}$, respectively, just before bypass was stopped. When fresh heparinized human blood was recirculated within an extracorporeal membrane oxygenator bypass circuit for 120 minutes, plasma β -TG rose to $5.13 \mu\text{g/mL}$, lactoferrin increased from 0.13 ± 0.04 to $1.62 \pm 0.22 \mu\text{g/mL}$, and neutrophil elastase rose from 0.05 ± 0.02 to $1.86 \pm 0.41 \mu\text{g/mL}$. At 120 minutes, lidocaine ($100 \mu\text{mol/L}$), which inhibits neutrophil activation, delayed release of lactoferrin ($1.33 \pm 0.26 \mu\text{g/mL}$) and markedly inhibited release of elastase ($0.24 \pm 0.05 \mu\text{g/mL}$) but did not inhibit release of β -TG antigen ($5.66 \mu\text{g/mL}$ at 120 minutes). PGE_1 ($0.3 \mu\text{mol/L}$) inhibited significantly the release of β -TG ($0.31 \mu\text{g/mL}$) and elastase ($0.52 \pm 0.11 \mu\text{g/mL}$) and attenuated the release of lactoferrin ($1.57 \pm 0.45 \mu\text{g/mL}$).

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DURING CARDIOPULMONARY BYPASS, blood anticoagulated with heparin leaves the normally non-thrombogenic endothelial-lined vascular system and comes in contact with synthetic surfaces of the extracorporeal circuit. Contact with synthetic materials leads to major qualitative and quantitative alterations in platelets that result in released granule contents, prolonged bleeding time, and increased blood loss.¹⁻³ During in vitro recirculation, thrombocytopenia,^{1,2} decreased sensitivity of platelets to aggregating agents,³ decreased α_2 -adrenergic,⁴ and fibrinogen receptors,⁵ secretion of thromboxane B_2 ,⁶ and depletion of α -granule contents³ have been observed.

The release of the lysosomal hydrolase β -glucuronidase during simulated extracorporeal circulation (SECC),⁷ as well as N-acetyl- β -glucosaminidase and β -glucuronidase, during clinical cardiopulmonary bypass (CCB),⁸ suggests neutrophil activation. However, neither of these enzymes are specific for neutrophils and also might be derived from either

platelets⁹ or monocytes.¹⁰ In this study, neutrophil activation was examined in both CCB and SECC by measuring two neutrophil proteins; lactoferrin, a specific granule marker, and human neutrophil elastase (HNE), an azurophilic granule marker.^{11,12} Characterization of the neutrophil response was addressed by observing the inhibitory effect of lidocaine and prostaglandin E_1 (PGE_1) on the release of these proteins in an extracorporeal model.¹

MATERIALS AND METHODS

CCB perfusion circuits contained a centrifugal pump (Biomedicus, Eden Prairie, MN), bubble oxygenator (BOS-102; Bentley Labs, Irvine, CA), and arterial line filter (model 1331) and cardiotomy reservoirs (Extracorporeal, Valley Forge, PA). SECC perfusion circuits (surface area of 0.9 m^2) including a spiral coil membrane oxygenator model 0800-2A (surface area of 0.8 m^2) and a venous reservoir (model R-500-4), were obtained from Sci-Med Life Systems, Minneapolis, and silicone tubing was obtained from Dow Chemical, Midland, MI. Beef lung heparin was obtained from Upjohn, Kalamazoo, MI; PGE_1 and N-formyl-L-Methionine-L-Leucyl-L-Phenylalanine (FMLP) from Sigma Chemical, St Louis; and lidocaine from Elkins-Sinn, Cherry Hill, NJ. Flat-bottomed 96 well polystyrene microtiter plates (Immulon no. 2) were obtained from Dynatech Laboratories, Alexandria, VA. Goat antirabbit IgG (heavy and light chains) covalently coupled to biotin and peroxidase-conjugated avidin as well as peroxidase-conjugated goat antirabbit IgG (heavy and light chains) were obtained from Cappel Laboratories, West Chester, PA.

Purified blood proteins. β -Thromboglobulin antigen (β TG) was purified to homogeneity as previously described.¹³ Purified human lactoferrin and monospecific rabbit antihuman lactoferrin were obtained from Calbiochem-Behring, La Jolla, CA. HNE was purified to homogeneity as described.¹⁴ Antibody raised in rabbits to HNE produced one precipitin arc either by immunodiffusion or immunoelectrophoresis against either purified HNE or a crude neutrophil extract. The HNE immunoassay utilized a 35% ammonium sulfate-enriched IgG fraction that was exhaustively dialyzed.

Clinical cardiopulmonary bypass. In ten patients from whom written informed consent was obtained, lactoferrin and HNE levels

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were measured in plasma samples obtained before, during, and after open-heart surgery for valvular or ischemic heart disease. In four of these patients plasma levels of platelet β TG were also sampled at the same time intervals. Blood samples (15 mL) were drawn from a radial artery catheter: (a) before induction of anesthesia; (b) after anesthesia, before heparin; (c) after heparin, before CCB; (d) five minutes after start of CCB; (e) 45 minutes after start of CCB; (f) just before CCB ended; (g) 60 minutes after CCB; and (h) 24 hours after CCB. All patients had CCB with a bubble oxygenator, centrifugal pump, and arterial line filter and were cooled to 26 °C to 28 °C during the procedure. Cold crystalloid potassium cardioplegia was used. Blood aspirated from the operative field was diluted in saline and centrifuged in a Dideco cell sorter (Mironcola, Italy) at 2,800 g and infused into each patient as packed cells.

Blood samples were collected into 1/10 volume sodium citrate (final concentration 0.38%) containing PGE₁ (final concentration 0.3 μ mol/L to prevent in vitro cell activation. Three milliliters were prepared as previously described for β TG measurements.¹³ Twelve milliliters were centrifuged at 2,225 g for 15 minutes at 23 °C. The supernatant was then recentrifuged at 27,500 g for 15 minutes and the resulting supernatant frozen at -70 °C in 0.8-mL aliquots for subsequent measurements of lactoferrin and HNE.

Simulated extracorporeal circulation. Perfusion circuits were assembled from standard silicone rubber components, polycarbonate connectors, and spiral coil membrane oxygenators as described previously.¹ Blood and gas compartments were flushed with 100% carbon dioxide for 15 minutes prior to priming the circuit. Oxygenators were primed by applying a vacuum to the lower gas port. Five hundred milliliters of blood were drawn from random donors who had abstained from any medications for 2 weeks directly into circuit venous reservoirs containing heparin (5.0 U/mL blood), glucose (3.3 mg/mL blood) and, in selected experiments, PGE₁ (0.3 μ mol/L) or lidocaine (100 μ mol/L) (final concentration in whole blood). PGE₁ (10 mg) was first dissolved in 0.01 mL of 95% ethanol and diluted with 0.2% Na₂CO₃ so that the total volume of PGE₁ solution added was 0.05 mL. Blood was permitted to enter the reservoir by gravity without foaming, and any air bubbles were removed from the system before starting the recirculation by a precisely shimmed, barely occlusive pump at a rate of 1.0 L/min for various lengths of time up to 120 minutes. The oxygenators were ventilated with a 95% oxygen-5% carbon dioxide mixture at a rate of 2.4 L/min. Blood temperature was maintained at 37 °C by immersing the reservoir bag in a constant-temperature water bath.

Samples of blood for analysis were collected either directly from the donor or from the reservoir at various times during recirculation. A sample of blood taken from the donor was incubated at 37 °C for 120 minutes and served as a standing control. Plasma samples were prepared as described above.

Blood cell counts. Whole blood platelet counts¹⁵ and leukocyte counts¹⁶ were obtained using a hemocytometer under phase microscopy. A differential leukocyte count was performed to assess the percent of neutrophils in the total population. Hematocrits were measured on each sample, and all counts expressed were corrected for by the calculated hemodilution.

Radioimmunoassay for β TG. Plasma β TG levels were measured as described previously from this laboratory.¹³

Enzyme-linked immunosorbent assay for HNE. Immunochemical measurement of total HNE concentration was performed by an indirect competitive enzyme-linked immunosorbent assay (ELISA) in 96-well microtiter plates as previously described,¹⁷ with the following modifications. Goat antirabbit IgG, conjugated with biotin, was added to each plate and incubated for 60 minutes at 23 °C, the plates were washed, and peroxidase-avidin was added for 30 minutes at 23 °C. The plates were again washed, substrate was

added; plates were incubated for 40 to 60 minutes, and the absorbance was measured at 450 nm.

ELISA for lactoferrin. Plasma lactoferrin levels were measured as described previously from this laboratory.¹⁸

Neutrophil isolation and aggregation. Neutrophils were isolated and aggregation was measured with a platelet aggregometer (Chronolog, Havertown, PA) as described previously from this laboratory.¹⁸ Neutrophils (1×10^7 /mL) were preincubated for 10 minutes at 37 °C with either PGE₁ (0.3 μ mol/L) or lidocaine (100 μ mol/L). They were then challenged with the chemotactic peptide FMLP (100 nM) and extent of aggregation measured. The results were compared to neutrophils that had not been exposed to either drug.

Lidocaine measurements. Plasma levels of lidocaine were measured at 0, 2, 10, 60, and 120 minutes after the start of recirculation, employing an immunoassay lidocaine kit obtained from SYVA, Palo Alto, CA.

Statistical analysis. Student's *t* test (paired) was used to test for significant differences between treatments at the end of SECC at 120 minutes. In all cases $\pm$ values stated indicate the mean $\pm$ the SEM. Analysis of variance was performed using all values for the data on the last three figures. All values listed as significant have a less than 5% occurrence by chance alone.

RESULTS

Clinical cardiopulmonary bypass. Following heparin administration, prebypass platelet counts fell to $92\% \pm 6\%$ of the initial platelet level. By five minutes into bypass, platelet counts had decreased to $40\% \pm 4\%$ (Fig 1) and remained at baseline levels for the duration of bypass. However, plasma β TG rose throughout CCB (Fig 1).

After heparin administration, prebypass neutrophil counts fell to $90\% \pm 4\%$ of the initial level and by 45 minutes neutrophil counts dropped to $67\% \pm 7\%$. However, by 60 minutes postbypass the neutrophil counts had returned to $103\% \pm 6\%$ of the initial neutrophil level (Fig 2). Prebypass

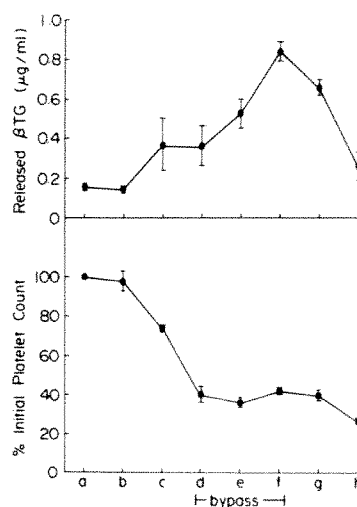


Fig 1. Platelet counts and plasma levels of platelet β TG during CCB. The percent initial platelet count and released β TG was determined: (a) before induction of anesthesia; (b) after anesthesia, before heparin; (c) after heparin, before CCB; (d) five minutes after start of CCB; (e) 45 minutes after start of CCB; (f) just before CCB ended; (g) 60 minutes after CCB; (h) 24 hours after CCB. Each point represents the mean $\pm$ SEM of four patients.

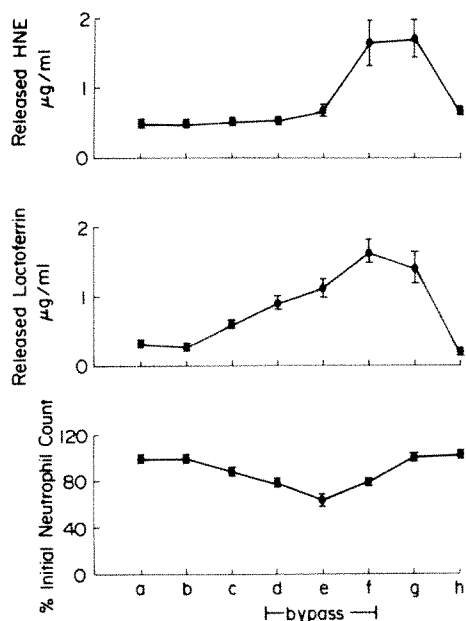


Fig 2. Neutrophil counts, plasma levels of neutrophil lactoferrin, and plasma levels of HNE during CCB. The percent initial neutrophil count, released lactoferrin, and released HNE were determined at the same time intervals as in Fig 1. Each point represents the mean $\pm$ SEM of ten patients.

lactoferrin levels rose from a baseline of $0.06 \pm 0.02 \mu\text{g/mL}$ to $0.56 \pm 0.17 \mu\text{g/mL}$ before bypass. During CCB, lactoferrin levels continued to rise steadily to a maximum of $1.66 \pm 0.33 \mu\text{g/mL}$ just before the end of CCB. However, lactoferrin concentrations fell to $1.45 \pm 0.48 \mu\text{g/mL}$ 60 minutes after bypass and by 24 hours had returned to an initial prebypass level of $0.23 \pm 0.06 \mu\text{g/mL}$ (Fig 2). In contrast, the prebypass levels of HNE did not change. After five minutes of bypass, HNE levels began to increase from a baseline of $0.49 \pm 0.07 \mu\text{g/mL}$. HNE levels rose throughout bypass to $1.65 \pm 0.68 \mu\text{g/mL}$. By 60 minutes, the HNE was still maximal at $1.71 \pm 0.55 \mu\text{g/mL}$ but returned to a value of $0.71 \pm 0.07 \mu\text{g/mL}$ by 24 hours after bypass (Fig 2).

Simulated extracorporeal circulation. During recirculation the circulating platelet count declined within two minutes and slowly increased over the next two hours, as has been reported previously.¹⁹ A steady rise in plasma levels of βTG to $5.13 \mu\text{g/mL}$ at 120 minutes (Fig 3) was observed. Addition of PGE_1 ($0.3 \mu\text{mol/L}$) completely preserved the prebypass platelet counts¹⁹ and prevented βTG release (Fig 3), while lidocaine ($100 \mu\text{mol/L}$) showed no effect on the release of βTG ($5.66 \mu\text{g/mL}$) at 120 minutes (Fig 3).

During SECC, neutrophil counts declined after 30 minutes, but even at 120 minutes the value was $81\% \pm 21\%$ of baseline. Neither PGE_1 ($0.3 \mu\text{mol/L}$) nor lidocaine ($100 \mu\text{mol/L}$) resulted in significant preservation of circulating neutrophil counts (Fig 4).

During SECC there was a steady rise in the plasma levels of lactoferrin from a baseline of $0.13 \pm 0.04 \mu\text{g/mL}$ to $1.62 \pm 0.22 \mu\text{g/mL}$ at 120 minutes. This level of lactoferrin at 120 minutes differed significantly from the level of $0.26 \pm 0.13 \mu\text{g/mL}$ ($P < .05$) found in the control maintained at 37°C

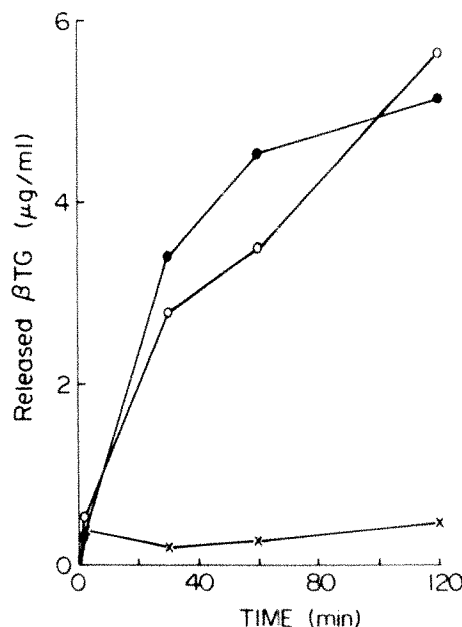


Fig 3. Plasma levels of platelet βTG in recirculated blood during SECC. Released βTG is expressed as μg of βTG per mL of plasma. Values were obtained when heparin anticoagulated blood was recirculated in the presence of PGE_1 ($0.3 \mu\text{mol/L}$) (X-X), lidocaine ($100 \mu\text{mol/L}$) (O-O), or absence of additional drug (●-●). Each point represents the mean of two separate determinations for each drug.

for 120 minutes without exposure to the circuit. PGE_1 ($0.3 \mu\text{mol/L}$) decreased the rate but did not completely inhibit lactoferrin released from neutrophil-specific granules (Fig 5). Lidocaine ($100 \mu\text{mol/L}$) appeared to show an inhibitory effect on the release of lactoferrin for the initial 20 minutes of perfusion (Fig 5). Although the $t_{1/2}$ of PGE_1 is known to be several hours in plasma,²⁰ the $t_{1/2}$ of lidocaine has not been previously examined. To estimate how much lidocaine was present in our circuit, we measured lidocaine levels in our samples and determined the $t_{1/2}$ of its disappearance to be 7 minutes. Since lidocaine was only introduced at the beginning of SECC, lidocaine levels would be 12% of that of the initial concentration at 21 minutes.

During recirculation there was a steady rise in plasma levels of HNE from a baseline of $0.05 \pm 0.02 \mu\text{g/mL}$ to $1.86 \pm 0.41 \mu\text{g/mL}$ at 120 minutes. This level of HNE when analyzed at 120 minutes differed significantly from the level of $0.37 \pm 0.06 \mu\text{g/mL}$ HNE ($P < .05$) found in the 120-minute standing control not exposed to the circuit. In contrast to the effect on either neutrophil count or lactoferrin

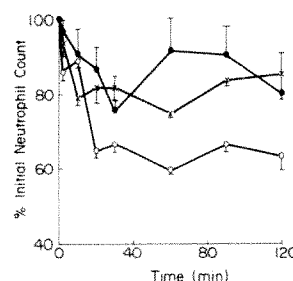


Fig 4. Neutrophil counts in recirculated blood during SECC. Neutrophil counts are expressed as percent initial control neutrophil count. The same symbols are used as in Fig 3. Each point represents the mean $\pm$ SEM of seven separate determinations.

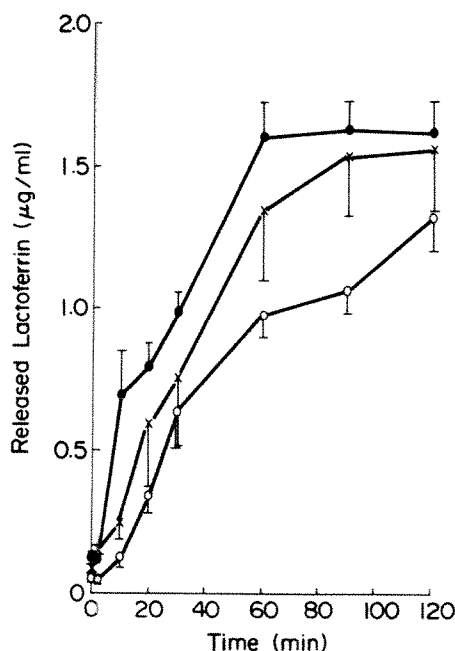


Fig 5. Plasma levels of neutrophil lactoferrin in recirculated blood during SECC. Released lactoferrin is expressed as μg of lactoferrin per mL of plasma. The same symbols are used as in Fig 3. Each point represents the mean $\pm$ SEM of seven separate determinations.

release, analysis of variance indicates a significant inhibition of the release of HNE during recirculation by both PGE_1 and lidocaine respectively (Fig 6).

To ascertain whether the inhibition of neutrophil degranulation observed in SECC was due to a direct effect on the cells, neutrophils were preincubated for ten minutes at 37°C with either PGE_1 ($0.3 \mu\text{mol/L}$) or lidocaine ($100 \mu\text{mol/L}$). Both treatments inhibited the maximal extent of in vitro aggregation induced by the chemotactic peptide FMLP by 50% and 53%, respectively (data not shown).

DISCUSSION

Cardiopulmonary bypass is used in approximately 200,000 Americans of all ages each year. The procedure differs substantially from hemodialysis in that the entire cardiac output in CCB passes through multiple components of the perfusion system, whereas in hemodialysis only a small (5% to 10%) portion passes through. The procedure is technically complex and varies in detail from patient to patient. With rare exception the patient's blood is diluted with crystalloid solutions yielding hematocrits of 20 to 25, cooled to 26°C to 28°C , and continuously filtered, washed, centrifuged, and returned to the patient as packed RBCs. In addition, patients receive a wide variety of drugs, anesthetic agents, and occasional blood transfusions. Usually the heart is perfused with cold cardioplegic solution every 20 to 30 minutes, and this solution (typically totalling 1 to 2 L) returns to the perfusate. The extensive dilution and processing of autologous blood and the presence of the patient's metabolic and clearance mechanisms, frustrate attempts to completely define all variables of blood-surface interaction in

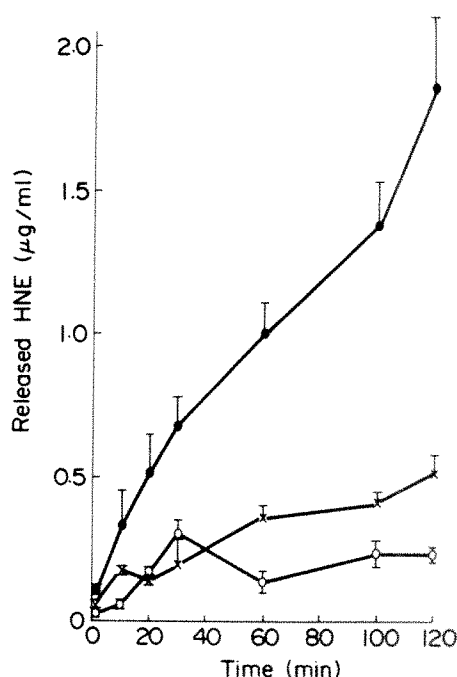


Fig 6. Plasma levels of HNE in recirculated blood during SECC. Released HNE is expressed as μg of HNE per mL of plasma. The same symbols are used as in Fig 3. Each point represents the mean $\pm$ SEM of seven separate determinations.

the clinical setting. However, because of possible clinical significance, it is important to verify the appearance and disappearance of various blood elements and cell-specific markers in vivo. Thus, this study for the first time identifies the appearance of lactoferrin and HNE in the circulation of patients who have undergone open-heart surgery.

SECC with fresh heparinized human blood permits qualitative measurements and careful study of the blood elements that directly interact with synthetic surfaces of the cardiopulmonary bypass system. Many of the variables that complicate the interpretation of in vivo results are eliminated. The use of heparin does not pose a problem with regard to neutrophil activation in SECC, since there is no significant rise in plasma levels of lactoferrin and HNE in 120-minute standing control samples that have not been exposed to the circuit. This model has proved particularly valuable in studies of platelet-surface interactions,²¹ and has led to measures to protect platelets during CCB.^{22,23}

CCB activates platelets³ and complement,²⁴ but heretofore activation of neutrophils with concomitant release of neutrophil-specific proteins has not been definitively demonstrated. Several investigators have demonstrated sequestration of neutrophils within capillaries during and immediately after cardiopulmonary bypass.²⁵ Appearance of both of the neutrophil-specific granule protein lactoferrin and azurophilic granule enzyme HNE in the plasma of both CCB and SECC indicates that neutrophils are stimulated and degranulation occurs.

Neutrophils contain many powerful lysosomal hydrolases as well as significant amounts of HNE, a major granular protease.^{11,12} Since the extracellular environment has a neu-

tral pH, released HNE has a potential for catalytic activation and may be important in mediating complications of CCB. HNE is a potent fibrinogenolytic and fibrinolytic enzyme,²⁶ that alters other coagulation proteinases,^{27,28} hydrolyzes platelet glycoprotein 1b,²⁹ reduces the number of thrombin-binding sites per platelet,³⁰ and exposes fibrinogen receptors on the platelet surface.³¹ Thus, HNE present in the extracellular environment after neutrophil degranulation could contribute to the hemorrhagic complications of prolonged CCB. In addition, because HNE attacks lung parenchyma³² and produces endothelial injury,³³ it may play a role in the capillary, pulmonary, and compartmental fluid changes associated with CCB. These changes could occur at HNE concentrations found in this study (Fig 2). When alpha-1-proteinase inhibitor is present, rapid inactivation of HNE occurs,³⁴ but oxidation of the critical Methionine residue of this plasma protease inhibitor may result in its inactivation allowing active HNE to act on connective tissues or other substrates.

Lactoferrin, located in the specific granules, is released at shorter incubation times and/or lower concentration of agonists than HNE.³⁵ It may be more difficult to inhibit lactoferrin exocytosis than HNE release. Lactoferrin may function as an autacoid potentiating other neutrophil agonists.¹²

SECC allows evaluation of agents that may modulate the ability of leukocytes and platelets to extrude their intracellular proteins. Lidocaine (100 $\mu\text{mol/L}$), a known inhibitor of neutrophils⁷ with a $t_{1/2}$ of seven minutes in this system, inhibits the release of lactoferrin for the first 20 minutes of recirculation, and then as the effect of lidocaine begins to wear off, lactoferrin gradually increases to levels seen in the absence of the drug (Fig 5). The concentration of lidocaine that has been used as an antiarrhythmic agent is 5 to 20 $\mu\text{mol/L}$.³⁶ In contrast, lidocaine virtually prevents the appearance of HNE (Fig 6) during SECC since lower concentrations are required for this effect. The effect of lidocaine is probably mediated directly since this compound inhibits neutrophil aggregation *in vitro*. Since we have not yet determined the minimal concentration necessary for inhibition of neutrophil exocytosis, its potential as a chemical inhibitor of neutrophil granule release cannot be ruled out. In contrast, βTG levels are not altered by lidocaine (Fig 3).

PGE_1 (0.3 $\mu\text{mol/L}$), a known inhibitor of platelet function,¹⁹ not only preserves the percent of initial platelet level and inhibits the release of βTG (Fig 3) as observed previously³⁷ but also affects neutrophil degranulation as reflected by the attenuated release of lactoferrin (Fig 5) and total inhibition of HNE degranulation (Fig 6) during SECC. PGE_1 has already been shown to inhibit both leukocyte aggregation in the pulmonary microcirculation³⁸ and granulocyte function.³⁹ We now demonstrate that PGE_1 directly inhibits FMLP-induced neutrophil aggregation, in agreement with the observation of Fantone et al⁴⁰ that PGE_1 decreased the generation of superoxide anion production and lysosomal enzyme secretion of neutrophils in response to FMLP. Additionally, recent work by Holcroft et al⁴¹ describes the ability of PGE_1 to inhibit inflammation in the lungs of patients with adult respiratory distress syndrome.

Although the percent of initial platelet levels during both SECC¹⁹ and CCB (Fig 1) decreases markedly, the percent of initial neutrophil levels falls to a lesser degree in either SECC (Fig 4) or CCB (Fig 2). Platelets adhere to synthetic surfaces of extracorporeal perfusion system^{1,38} and are activated to synthesize thromboxanes⁶ and release α -granule contents.³ Membrane α_2 -adrenergic⁴ and fibrinogen receptors⁵ are reduced, and remaining circulating platelets lose their ability to aggregate in response to established agonists.³ In contrast, the failure of neutrophil counts to decrease to the extent of that of the platelets during recirculation suggests that neutrophil adherence to the synthetic surfaces of the system is less sensitive as an activating mechanism than it is with platelets. Thus, the soluble neutrophil agonists that are generated as a result of activation of other blood elements and/or proteins on the surface of the extracorporeal circuit are implicated in neutrophil activation.

Complement activation occurs during CCB.²⁴ The formation of C5a, a potent neutrophil agonist,⁴² is inferred from observed increases in C3a.⁴³ No direct increase of C5a has as yet been measured, probably because any C5a generated is readily internalized by the neutrophil. Thus, the contribution of C5a as a neutrophil agonist is not yet clear in SECC.

Recent studies by Heller et al⁴⁴ have indicated that during CCB plasma prekallikrein levels significantly decrease, and the generation of kallikrein-like activity has been observed. Plasma kallikrein has been shown to induce neutrophil chemotaxis,⁴⁵ aerobic glycolysis,⁴⁶ aggregation,^{47,48} and degranulation^{17,46} and may serve as a direct agonist in this system. Additionally, kallikrein activates Factor XII to Factor XIIa,^{49,50} which recently has been shown to aggregate and stimulate exocytosis of HNE from neutrophils.¹⁸ Very little is known about the contact system enzymes during CCB, and further investigation is needed.

In this study we have observed increased plasma levels of βTG in both SECC (Fig 3) and CCB (Fig 1). Platelet-specific proteins such as βTG or platelet-derived growth factor may serve as potential neutrophil agonists. Recent studies indicate that other platelet-specific α -granule proteins, platelet Factor 4⁵¹ and platelet-derived growth factor induce neutrophil chemotaxis.⁵² In addition, the formation of leukotrienes, potent autacoids for neutrophils, may also play a role in this activation process. Any or all of the aforementioned neutrophil agonists could singularly or in concert at lower concentrations be responsible for the observed neutrophil activation and subsequent degranulation that we have documented to occur during both SECC and CCB. Identification of the responsible humoral substances would allow for rational and specific intervention during CCB to prevent neutrophil alterations and their potential deleterious consequences during CCB.

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Immunogenic Nature of a *Pol* Gene Product of HTLV-III/LAV

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The present studies were initiated to define the coding region of a 34 kilodalton (kd) protein (p34) frequently observed with antibodies from HTLV-III/LAV-infected people by immunoblotting and radioimmunoprecipitation (RIP) techniques. We have directly mapped this viral protein to the *pol* gene of HTLV-III/LAV by radiolabeled amino acid sequence analysis. This region at the 3' end of the *pol* gene is predicted to encode the endonuclease/integrase of the virus. The seroprevalence rate of antibodies to the *pol* gene products p64 and p53 and to the endonuclease p34 were evaluated. Of 161 HTLV-III/LAV seropositive people tested by immunoblotting procedures, greater than 98% had antibodies which reacted to p64/p53 and 92.6%

reacted to p34 indicating that these viral proteins are highly immunogenic in nature. We have also analyzed the serum of nine healthy people living in West Africa who were infected with HTLV-IV, a closely related retrovirus. Nine of nine seropositive people had antibodies that cross-reacted to p34 of HTLV-III/LAV, whereas only seven of nine reacted to p64/p53. These studies and our earlier observations indicate that current diagnostic procedures for screening for HTLV-III/LAV infection may also detect HTLV-IV seropositive individuals, pointing to a need for more specific assay systems.

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THE RETROVIRUS responsible for the acquired immune deficiency syndrome (AIDS) termed HTLV-III/LAV or ARV contains an array of potential genes in addition to the classical *gag*, *pol*, and *env* genes of most retroviruses.¹⁻⁴ Since the virus was first isolated much effort has focused on the identification of these viral proteins. Of particular interest has been the observation of variations in serologic profiles of infected people and potential use of these patterns as indicators of disease progression. Previous studies have shown that the *env*-encoded proteins gp160 and gp120 are the most immunogenic proteins irrespective of disease state, whereas the prevalence of antibodies to p24, the major core antigen, decline with disease.^{5,6} As the gene products are identified more information has accumulated as to these serologic variations. Previous studies have identified two proteins, p64 and p53, as encoded by the reverse transcriptase region of the *pol* gene of HTLV-III/LAV.⁷ In addition, indirect evidence has suggested that a 31- to 34-kd protein was encoded by the 3' end of *pol* using bacterially expressed products in a competition immunoblot assay.^{8,9}

Recently a closely related simian retrovirus was isolated from healthy African green monkeys and termed STLV-III.¹⁰ This virus shares many characteristics with HTLV-III/LAV including cell tropism, ultrastructural morphology, and similar protein characteristics. A new human T-lymphotropic virus infecting healthy persons in West Africa shows many of the same characteristics as STLV-III and is indistinguishable serologically.^{11,12} This virus has been named HTLV-IV and immunological cross-reactivity with HTLV-III/LAV has been shown by immunoblotting and radioimmunoprecipitation (RIP) procedures. This has important implications as new diagnostic screening procedures are developed to accurately detect infection by HTLV-III/LAV or a related virus such as HTLV-IV. Here we report the identification of the putative endonuclease/integrase of HTLV-III/LAV, p34, assess the immunogenicity of this viral protein in infected people, and finally show that this protein contains conserved antigenic determinants between HTLV-III/LAV and HTLV-IV.

MATERIALS AND METHODS

The HTLV-IIIB/MOLT-3 cell line was used for antigen preparations (gift from Dr Robert Gallo). Immunoblotting procedures and

methods for virus purification were carried out as previously described.¹¹ Radioimmunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis (RIP, SDS-PAGE) were carried out as described.⁵ Cells were radiolabeled with [35-S] cysteine, [35-S] methionine, or [3-H] amino acids in appropriate labeling media for eight hours. The solubilized viral antigens were immunoprecipitated, the precipitates were washed, and resulting protein was electrophoresed on 12.5% SDS gels. Techniques for radiolabeled amino-terminal amino acid sequencing were performed as described.⁶ Radiolabeled viral antigens were immunoprecipitated, separated on SDS gels, electroeluted, dialyzed, and subjected to Edman degradation.

RESULTS

An example of the serum profiles obtained by immunoblotting procedures is shown in Fig 1A. Represented is a healthy laboratory HTLV-III/LAV seronegative person (lane 1), two healthy homosexual seronegatives (lanes 2 and 3), three healthy HTLV-III/LAV seropositives (lanes 4 through 6), three AIDS-related complex (ARC) patients (lanes 7 through 9), and three AIDS patients (lanes 10

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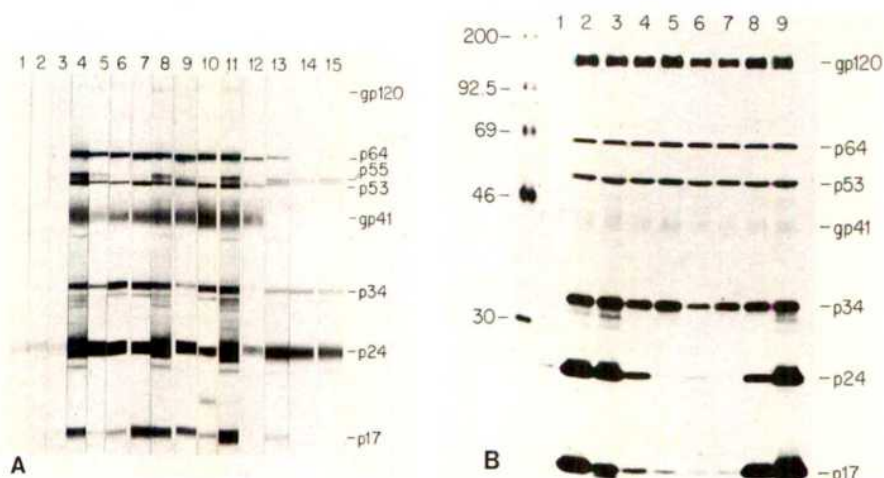


Fig 1. Reactivity of antibodies to HTLV-III/LAV specific p34. (A) Western blotting of HTLV-III/LAV antigens. The antigens from SDS-polyacrylamide gels were blotted onto nitrocellulose sheets and test strips reacted with a 1:100 dilution of serum from the following: A laboratory worker (lane 1); healthy homosexual males (lanes 2 through 6); ARC patients (lanes 7 through 9); AIDS patients (lanes 10 through 12); and HTLV-IV-infected healthy West Africans (lanes 13 through 15). (B) Radioimmunoprecipitation (RIP) of [35-S] cysteine- and [35-S] methionine-labeled virus. The antigens were solubilized and immunoprecipitated with the following: A laboratory worker (lane 1), and eight HTLV-III/LAV seropositive individuals (lanes 2 through 9).

through 12). Serum from all seropositive individuals recognize a 34-kd protein (p34). A comparison with RIP/SDS-PAGE is shown in Fig 1B. In every seropositive case shown, a p34 species was observed using this procedure.

To ascertain whether this protein was encoded by the genome of HTLV-III/LAV, radiolabeled sequence analysis was performed. p34 was individually radiolabeled with [3-H] phenylalanine, isoleucine, and lysine, isolated by SDS-PAGE, and subjected to amino-terminal amino acid sequencing. The results are shown in Fig 2. Phenylalanine peaks were observed as positions 1 and 26, lysine peaks were seen at 7 and 14, and an isoleucine peak was seen at position 5 of the p34 sequence. Analysis of the predicted amino acid sequence from the *pol* gene region indicates that p34 maps to the 3' end of this gene and begins at nucleotide sequence 4263 of the HTLV-III/LAV genome as published by Ratner et al.¹ The predicted amino acid sequence is shown in Fig 2B.

The probability of a protein with a random amino acid sequence having lysines, phenylalanines, and an isoleucine in these positions is less than 5.5×10^{-9} .

We analyzed 205 serum samples from people at high risk for infection and those with evidence of HTLV-III/LAV-related disease for the seroprevalence of p34 (Table 1). Of these samples, 161 were positive for antibodies to HTLV-III/LAV proteins by immunoblotting. Overall, 92.6% of seropositives had antibodies to p34, whereas the seroprevalence ratio to p64/p53 was greater than 98%, higher than any other category of protein including the *env*-encoded gp41. It should be noted, however, that all seropositives detected gp120 and gp160 by RIP/SDS-PAGE procedures. Prevalence of antibodies to reverse transcriptase p64/p53 is high irrespective of disease state, but the prevalence to p34 drops slightly in the AIDS category. Additionally, we examined nine representative serum samples from HTLV-IV-

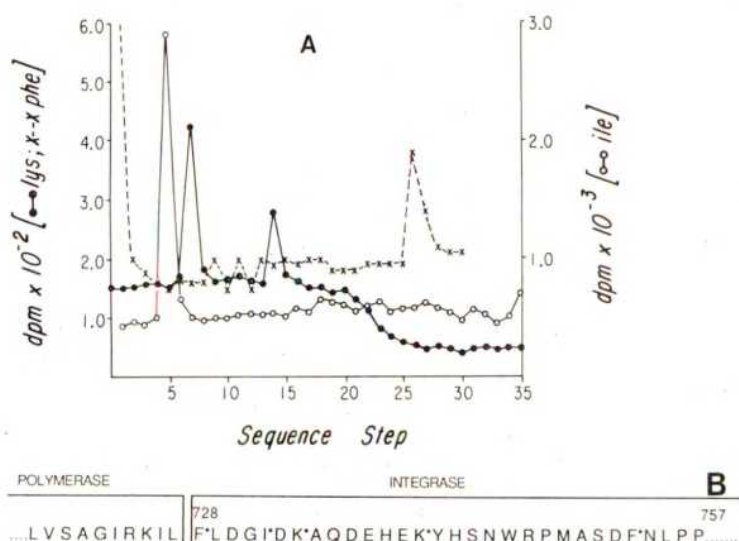


Fig 2. (A) Amino acid sequence analysis of the amino-terminus of p34. The protein was labeled with [3-H] phenylalanine, isoleucine, or lysine and subjected to Edman degradation; radioactivity is shown for each degradation cycle. (B) Alignment of the amino acid sequence of p34 to the predicted amino acid sequence from the 3' end of *pol*. Asterisks represent radiolabeled amino acids and their positions from the amino-terminus of p34. The numbers refer to the amino acid position relative to the first sequence of the *pol* open reading frame.

Table 1. Prevalence of Antibodies to HTLV-III/LAV Viral Proteins by Disease State as Detected by Western Blotting

Category	gp41	p64	p53	p34	p24
Healthy	37(90.2)	41(100)	40(97.5)	39(95.1)	41(100)
ARC	67(100)	67(100)	67(100)	66(98.5)	64(95.5)
AIDS	53(100)	52(98.1)	52(98.1)	44(83.0)	50(94.3)
TOTAL	157(97.5)	160(99.4)	159(98.7)	149(92.6)	155(96.8)

Samples were selected at random from stored sera collected from area hospitals and clinics. Disease states were defined using CDC criteria. The numbers in parentheses reflect percentage positive based on the highest value for a given protein in that disease category. Forty additional samples were tested from healthy and ARC categories and were negative for antibodies to the above proteins. Total number of seropositives was 161 and was used as the denominator for percentage determinations.

infected people for antibodies to the *pol* gene products of HTLV-III/LAV. Three serum profiles are illustrated in Fig 1A, lanes 13 through 15. In addition to reactivity to the *gag*-related antigen p24 of HTLV-III/LAV, all nine samples recognized the p34 protein whereas only seven of nine detected p64.

DISCUSSION

As each new viral protein has been identified and its seroprevalence assessed, a better understanding of the humoral response to infection unfolds. The gene products can then be evaluated on the basis of their relative antigenicity. The present results indicate that the *pol* gene products which include the previously identified p64/p53 and the above-discussed endonuclease p34 are highly immunogenic in

HTLV-III/LAV-infected people. The most immunogenic proteins are the *env*-encoded glycoproteins gp160 and gp120, followed by *pol*-encoded p64/p53, *env*-encoded gp41, *gag*-encoded p24, and *pol*-derived p34. The relationship between serologic profiles and disease state has received much attention from investigators in hopes of finding key serologic markers indicative of clinical progression of disease. Unfortunately, no clear pattern has emerged although relative decreases in seroprevalence of p24, p17, and p34 have been observed in AIDS patients.

Perhaps the most intriguing aspect of the antigenic nature of these viral proteins relates to the high degree of immunologic cross-reactivity between HTLV-III/LAV and HTLV-IV. The significance of these findings is obvious when the design of second-generation screening assays is devised for detecting antibodies to HTLV-III/LAV. As previously reported, many HTLV-IV-infected people test positive by enzyme-linked immunosorbent assay (ELISA) to HTLV-III/LAV.¹¹ The exact nature of this ELISA cross-reactivity is unknown although limited cross-reactivity exists between *env*-encoded proteins for these viruses as well as the *gag*- and *pol*-derived proteins. Detailed analysis of cross-reactive epitopes is necessary, therefore, to devise assays that would include or exclude conserved epitopes. In the former case, inclusion of conserved epitopes would be helpful for screening for HTLV-III/LAV and related viruses in blood banking situations. In the latter case, nonconserved epitopes in an assay system would be preferred for delineating between HTLV-III/LAV and HTLV-IV exposure, which may then have clinical relevance.

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A Role for Guanine Ribonucleotides in the Regulation of Myeloid Cell Maturation

By Daniel G. Wright

We have shown previously that induced maturation of the human myeloid leukemia cell line, HL-60, is associated with a selective down-regulation of guanine ribonucleotide synthesis and depletion of intracellular guanosine triphosphate (GTP) and guanosine diphosphate (GDP) pools. We showed, furthermore, that inhibitors of the enzyme, inosine monophosphate (IMP) dehydrogenase, which catalyzes the initial rate-limiting step of guanylate synthesis from the central intermediate IMP, are potent inducers of myeloid maturation in these cells. We now show that induced maturation of HL-60 cells is prevented or impaired if intracellular concentrations of guanine ribonucleotides

are maintained at high levels. HL-60 cells can utilize exogenous guanine and guanosine to maintain GTP and GDP pools through a salvage pathway that bypasses guanylate synthesis from IMP. Moreover, incubation of HL-60 cells with guanosine or guanine (10^{-6} to 10^{-4} mol/L) prevents both the depletion of intracellular guanine ribonucleotides and the induction of myeloid maturation caused by the IMP dehydrogenase inhibitor, tiazofurin. These findings provide strong additional support for the concept that terminal myeloid differentiation is influenced by a guanine ribonucleotide-dependent regulatory system.

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A NUMBER OF HUMAN myeloid leukemia cell lines have been established which not only can be maintained as immature blast cells in continuous, self-renewing culture but can also be induced to undergo maturation in vitro.¹ These cell lines have attracted considerable interest, for they provide experimentally accessible model systems with which to define basic mechanisms involved in the regulation of myeloid progenitor cell proliferation and differentiation. We used the HL-60 cell line, originally isolated from a patient with promyelocytic leukemia,²⁻⁴ to define a role for intracellular guanine ribonucleotides in the regulation of myeloid cell maturation. As we reported previously, HL-60 maturation induced by *cis*-retinoic acid or dimethylformamide is associated with a depletion of intracellular guanosine triphosphate (GTP) and guanosine diphosphate (GDP), whereas adenylate pools remain relatively intact.⁵ This selective depletion of guanylates occurs in part because guanylate synthesis from the central intermediate, inosine monophosphate (IMP), is down-regulated during induced maturation at the rate-limiting step catalyzed by IMP dehydrogenase (IMPD).⁵⁻⁷ We also observed that specific inhibitors of IMPD [eg tiazofurin (RTC) or mycophenolic acid] are themselves potent inducers of HL-60 maturation.^{5,6} To explore further the apparent relationships between intracellular supplies of guanine ribonucleotides and myeloid maturation, we examined the possibility that induced maturation of HL-60 cells might be prevented or impaired if intracellular concentrations of guanine ribonucleotides are maintained at high levels.

MATERIALS AND METHODS

Maintenance and induced maturation of myeloid leukemia cells. Cryopreserved, early passage HL-60 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained by passage into fresh serum-free medium⁵ every 3 to 4 days. Cells were studied at maintenance passages 20 through 40. All cell incubations were in 75-cm² tissue culture flasks at 37 °C with 7.5% CO₂ and 100% humidity. Cell counts in culture specimens were determined by hemocytometer chamber counting, and cell viability was assessed by 0.1% trypan blue exclusion.

Cells in log-phase growth (2 to 3 days following media change) were induced to mature by inoculation at 3×10^5 cells/mL into fresh serum-free media supplemented with inducing compounds: 2- β -D-ribofuranosylthiazole-4-carboxamide (RTC or tiazofurin, obtained from the Drug Synthesis and Chemistry Branch, DCT, NCI, National Institutes of Health, Bethesda, MD), 1.0 μ mol/L; retinoic acid (Sigma Chemical, St Louis), 1.0 μ mol/L; or dimethylformamide (Eastman Kodak Co, Rochester, NY), 60 mmol/L. Induced maturation of the cells was assessed by methods described previously for determining NBT reduction^{5,8} and reactivity to the monoclonal antibody OK-M1 (Ortho Diagnostics, Raritan, NJ) as detected with FACS analysis.⁹ For certain studies, cells were exposed to inducing agents in incubation media supplemented with guanosine or guanine (Sigma). Guanosine and guanine were dissolved in sterile dH₂O to prepare stock solutions (10^{-2} mol/L), which were then further diluted in culture media to achieve final concentrations of 10^{-7} to 10^{-4} mol/L. Guanosine was readily soluble, whereas guanine at 10^{-2} mol/L required alkalization with 10 mol/L KOH, 8 μ L/mL dH₂O, to dissolve.

Measurement of intracellular purine ribonucleotide concentrations. Purine ribonucleotide concentrations in perchlorate (PCA) extracts of 2×10^7 cell pellets were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) using an anion-exchange, microparticulate column (Partisil-10 SAX, Whatman, Clifton, NJ) as described previously.⁵ Values for ATP and GTP concentrations (nmoles/ 10^7 cells) were derived from chromatographic peaks detected by ultraviolet (UV) absorbance at 254 nm which were related to standards chromatographed at known concentrations. The use of exogenous hypoxanthine, guanine, and guanosine for synthesis of ribonucleotides was assessed by incubating cells with [8-¹⁴C] hypoxanthine (49.0 mCi/mmol; 1.0 μ Ci/mL), [8-¹⁴C] guanosine (510.4 mCi/mmol; 1.0 μ Ci/mL), or [8-¹⁴C] guanine (55.5 mCi/mmol; 10.0 μ Ci/mL) (New England Nuclear, Boston) followed by PCA extraction of cell pellets and analysis by HPLC with simultaneous measurement of UV absorbance and radioactivity in eluants.⁵ Specific incorporation of radiolabel into ribonucleotides

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Table 1. Utilization of Hypoxanthine, Guanine, and Guanosine for the Synthesis of ATP and GTP by HL-60 Cells

Incubation Conditions	¹⁴ C-Hypoxanthine		¹⁴ C-Guanine		¹⁴ C-Guanosine	
	ATP	GTP	ATP	GTP	ATP	GTP
Control						
Nmoles/10 ⁷ cells	51.8	10.9	56.0	13.3	50.7	13.9
Incorporation of radiolabel*	54	70	26	299	33	188
RTC (10 ⁻⁶ mol/L):						
Nmoles/10 ⁷ cells	50.7	4.9	52.4	5.4	47.7	5.1
Incorporation of radiolabel*	47	2	20	992	28	659

HL-60 cells were cultured with or without 2-β-D-ribofuranosylthiazole-4-carboxamide (RTC) for 24 hours in serum-free media as previously described⁶; [8-¹⁴C] hypoxanthine (49.0 mCi/mmol; 0.2 μCi/10⁷ cells), [8-¹⁴C] guanosine (510.4 mCi/mmol; 0.5 μCi/10⁷ cells), or [8-¹⁴C] guanine (1.0 μCi/10⁷ cells; 55.5 mCi/mmol) were added to cell cultures and, after an additional 4 hours, ribonucleotides were extracted from cell pellets with perchlorate and analyzed by high-pressure liquid chromatography (HPLC).⁶ Results are representative of three separate studies; data are means of duplicate measurements, which in all cases varied from the mean by <10%.

*Incorporation of radiolabel = radioactivity (dpm × 10⁻²)/nmole of nucleotide detected in ATP and GTP peaks.

was expressed as radioactivity (dpm × 10⁻²)/nanomole which was calculated by dividing the total dpm (× 10⁻²) detected in a nucleotide peak by the amount of nucleotide (in nmoles) measured in that peak.

RESULTS

HL-60 cells, cultured in a completely defined, serum-free medium, actively salvaged hypoxanthine to generate purine ribonucleotides.⁵ As shown in Table 1, HL-60 cells could also be shown to utilize exogenous guanosine and guanine for ribonucleotide synthesis. Moreover, when these cells were incubated with the IMPD inhibitor tiazofurin (RTC), 10⁻⁶ mol/L, the relative utilization of radiolabeled guanosine or guanine for GTP synthesis was increased, whereas utilization of hypoxanthine for guanylate synthesis, which proceeds through IMP, was almost completely blocked, reflecting IMPD inhibition (Table 1).

As shown in Fig 1, incubation of HL-60 cells with RTC (10⁻⁶ mol/L) resulted in a substantial decrease in intracellular GTP concentrations along with a rise in the ratio of ATP:GTP, reflecting the relatively selective blockade of guanylate synthesis from IMP caused by this agent.^{5,6} However, the addition of guanosine or guanine (10⁻⁶ to 10⁻⁴ mol/L) to the culture medium prevented GTP depletion as well as the increased ATP:GTP ratio caused by RTC under control conditions. Moreover, guanosine and guanine in a similar dose-responsive manner also prevented the induction of myeloid maturation and the decline in cellular proliferation observed in control cell cultures treated with RTC alone (Fig 2). For the studies shown in Fig 2, NADPH oxidase activity, measured as phorbol diester-stimulated NBT reduction, was used as the marker of induced myeloid maturation. Very similar results were obtained, however, when acquisition of the OK-M1 antigen, measured by FACS analysis,⁹ was used to assess induced maturation.

Exogenous guanosine and guanine did not impair the activity of RTC as an IMPD inhibitor. Indeed, the almost complete blockade of hypoxanthine utilization for guanylate synthesis caused by RTC (Table 1) was even more evident when HL-60 cells were incubated with 10⁻⁴ mol/L of guanosine or guanine.

Although IMPD inhibition and guanine ribonucleotide depletion occur rapidly in cells incubated with RTC (within

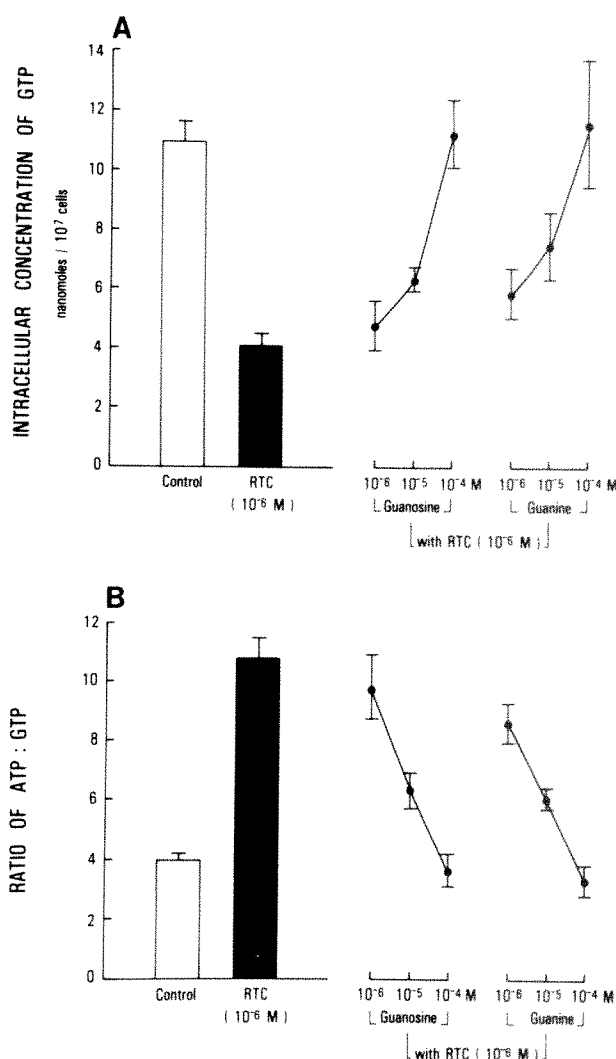


Fig 1. Reversal of the tiazofurin [2-β-D-ribofuranosylthiazole-4-carboxamide (RTC)]-induced depletion of GTP (A) and increased ratio of ATP:GTP (B) in HL-60 cells when incubation media was supplemented with guanosine or guanine (10⁻⁶ to 10⁻⁴ mol/L). Data are mean values from six replicate studies ± SEM.

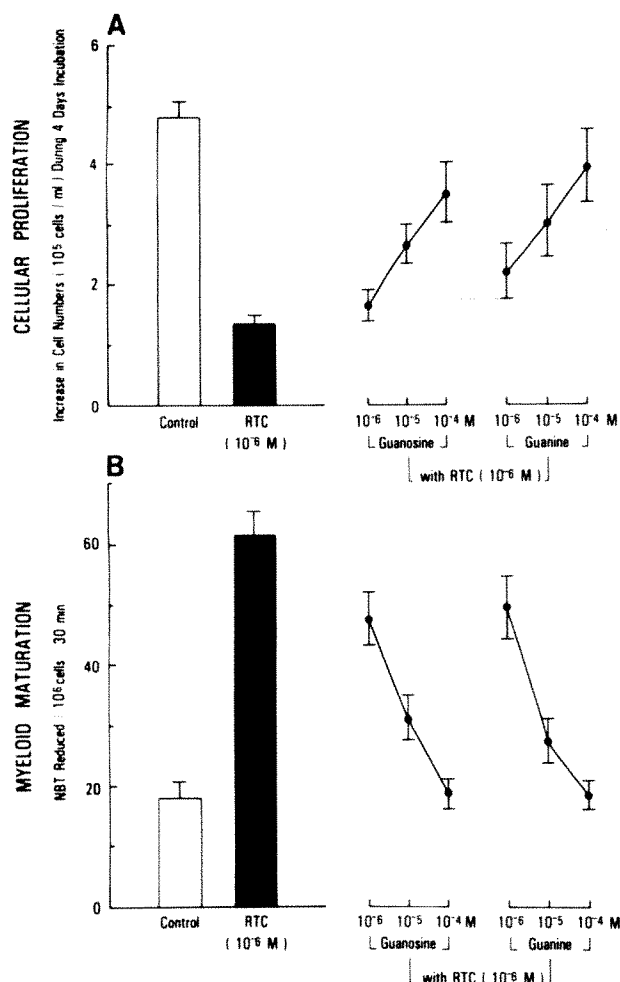


Fig 2. Reversal of the tiazofurin [2- β -D-ribofuranosylthiazole-4-carboxamide (RTC)]-induced inhibition of cellular proliferation (A) and myeloid maturation (B) of HL-60 cells when incubation media was supplemented with guanosine or guanine (10^{-6} to 10^{-4} mol/L). Data are mean values from six replicate studies $\pm$ SEM.

6 hours),⁷ the induction of maturation was found to be largely prevented (by >85%) if guanosine (10^{-4} mol/L) was added to the culture medium within 24 hours after incubation of cells with RTC had been started. This effect was progressively diminished if guanosine was added after 24

hours, however, and was inapparent following a delay of 72 hours.

Exogenous guanosine (10^{-4} mol/L) was also found to reverse the depletion of GTP pools in HL-60 cells incubated with retinoic acid (RA) or dimethylformamide (DMF) (Table 2). They also partially reversed the decline in cellular proliferation and the induction of myeloid maturation caused by these agents; however, these effects were not as complete as in HL-60 cells induced with RTC. In contrast to guanosine and guanine, exogenous inosine, adenosine, and hypoxanthine (10^{-4} mol/L) did not reverse the depletion of intracellular GTP or the induction of myeloid maturation observed in HL-60 cells incubated with RTC, RA, or DMF. This result is consistent with a blockade in the synthesis of guanylates from IMP that we have shown to occur in HL-60 cells induced to mature by RTC, RA or DMF.^{5,7} The utilization of inosine, adenosine, and hypoxanthine for guanylate synthesis involves overlapping salvage pathways, each of which ultimately proceeds through IMP, whereas the utilization of guanosine and guanine to form guanine ribonucleotides involves the direct salvage of guanine to GMP, bypassing the synthetic pathway from IMP.

DISCUSSION

Our previously reported observation that IMP dehydrogenase inhibitors are potent inducers of HL-60 cell maturation suggested that a guanine ribonucleotide-dependent system is involved in the regulation of induced myeloid cell maturation, since this enzyme is normally rate-limiting for the production of guanylates in cells. Our present studies, which show that the maturation-inducing effects of selective IMPD inhibition can be completely blocked if intracellular supplies of guanine ribonucleotides are maintained at high levels, provide strong additional support for this idea.

Both exogenous guanosine and guanine were effective in preventing guanine ribonucleotide depletion in cells treated with the IMP dehydrogenase inhibitor, tiazofurin (RTC). Because human cells have been found to lack guanosine kinase activity by which guanosine might be converted directly to GMP, it is presumed that guanosine is first metabolized to guanine by nucleoside phosphorylase and then salvaged directly through a guanine phosphoribosyl transferase-mediated step to form GMP. Thus, both guano-

Table 2. Effects of Exogenous Guanosine and Guanine on HL-60 Cells Exposed to Retinoic Acid and Dimethylformamide

Incubation Conditions	Intracellular (GTP) (nmoles/10 ⁷ Cells)	Cellular Proliferation (Percentage of Increase in Cell Concentration by 4 Days)	Myeloid Maturation (NBT Reduction ΔOD_{515})
Control	11.3 $\pm$ 0.9	157 $\pm$ 13	18 $\pm$ 4
RA (10 ⁻⁶ mol/L)	7.0 $\pm$ 0.5	50 $\pm$ 3	196 $\pm$ 12
+ Guanosine (10 ⁻⁴ mol/L)	11.5 $\pm$ 1.5	100 $\pm$ 30	110 $\pm$ 12
+ Guanine (10 ⁻⁴ mol/L)	10.5 $\pm$ 2.0	87 $\pm$ 7	122 $\pm$ 14
DMF (60 mmol/L)	5.2 $\pm$ 0.7	83 $\pm$ 13	107 $\pm$ 17
+ Guanosine (10 ⁻⁴ mol/L)	8.5 $\pm$ 1.5	150 $\pm$ 30	55 $\pm$ 6
+ Guanine (10 ⁻⁴ mol/L)	8.5 $\pm$ 0.8	133 $\pm$ 17	65 $\pm$ 8

HL-60 cells were cultured with and without retinoic acid (RA) or dimethylformamide (DMF), with and without guanosine or guanine for 4 days in serum-free media. Measurements of cellular proliferation represent percentages of increase in cell concentrations during 4-days culture from an initial seeding concentration of 3×10^5 /mL (percentage of increase = absolute increase in cell concentration ($\times 10^5$ /mL) by 4 days/ 3.0×10^5). Measurements of myeloid maturation represent NBT-reduced per 10^6 cells during 30-minute incubation with 100 ng/mL of phorbol myristate acetate.^{6,9} Results are mean values $\pm$ SEM from four replicate studies.

sine and guanine are available as precursors for guanine ribonucleotide synthesis by a final salvage reaction that bypasses the principal synthetic pathways which proceed by IMP. Both guanosine and guanine were examined because of the possibility that they might not be equally accessible for entry into the cells; however, both were similarly effective at comparable concentrations in preventing GTP depletion in induced cells and in preventing induced maturation.

These studies are the first to define a relationship between intracellular guanine ribonucleotide concentrations and cellular maturation in a eukaryotic cell. Such a relationship has been described previously in prokaryotes, however. Guanine nucleotide deprivation induces sporulation of *Bacillus subtilis* and *Sacharomyces cerevisiae* in a manner analogous to the induced maturation of HL-60 cells. Not only do inhibitors of guanylate synthesis (including IMPD inhibitors) trigger the sporulation of these microorganisms, but exogenous guanine, sufficient to protect intracellular guanine ribonucleotide pools, prevents sporulation.^{10,11} The guanine ribonucleotide-dependent system involved in controlling sporulation has not been defined; however, it has been suggested that a GTP-dependent repressor protein may be associated with controlling regions of DNA that directly regulate gene expression relevant to microbial sporulation.¹¹

It is similarly tempting to speculate that guanylate depletion and repletion may influence myeloid maturation in HL-60 cells by affecting the activity of a GTP-binding regulatory protein or proteins. Guanine nucleotide binding proteins are known to regulate a variety of critical cellular

activities, including the ribosomal assembly of polypeptides and receptor mediated responses to extracellular stimuli.¹²⁻¹⁴ The involvement of such GTP-binding proteins in the regulation of hormone-sensitive adenylate cyclase has been particularly well defined,^{13,14} and it is of interest with regard to our current studies that the same experimental maneuvers, which we find to affect both intracellular guanine ribonucleotide concentrations and the induction of myeloid maturation in HL-60 cells, have been shown to affect the activation of adenylate cyclase in intact rat kidney cells by isoproterenol.¹⁵ Hence, it is not unreasonable to suggest that depletion and repletion of intracellular guanine ribonucleotides in HL-60 cells may affect the function of a guanine nucleotide binding protein or proteins involved in regulating the responsiveness of the cells to hormonal signals. Basic mechanisms underlying the neoplastic transformation of HL-60 cells have not yet been defined; however, these mechanisms may involve an autocrine growth hormone or amplified responses by the cells to growth hormones. In either case, IMPD inhibition and guanine ribonucleotide depletion may interfere with the cells' ability to respond to proliferative signals by affecting a "G-protein"-regulated transduction pathway for such signals, thereby triggering cellular maturation as a permissive event.

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A Monoclonal Antibody to the Membrane Glycoprotein Complex CD18 Inhibits Polymorphonuclear Leukocyte Accumulation and Plasma Leakage In Vivo

By Karl-E. Arfors, Claes Lundberg, Lennart Lindbom, Kerstin Lundberg, Patrick G. Beatty, and John M. Harlan

Previous *in vitro* findings suggest a critical role for the polymorphonuclear leukocyte (PMN) membrane glycoprotein complex CD18 in PMN adherence and chemotaxis. We examined the effect of the murine monoclonal antibody (MoAb) 60.3, recognizing CD18, on induced PMN accumulation *in vivo*. Rabbits were pretreated with MoAb 60.3, and the chemotactic factors fMLP, leukotriene (LT)_{B₄}, and C5a, as well as histamine, were injected intradermally; 4 hours later, plasma leakage (¹²⁵I-albumin) and the PMN accumulation (myeloperoxidase) were determined. Both PMN accumulation and PMN-dependent plasma leakage were abolished in the inflammatory skin lesions of rabbits

pretreated with MoAb 60.3 as compared with control animals, whereas histamine-induced PMN-independent plasma leakage was unaffected. Intravital microscopy of the rabbit tenuissimus muscle revealed that MoAb 60.3 inhibited both PMN adherence in the venules and migration into the tissue following application of LT_{B₄} and zymosan-activated serum (ZAS). Rolling of PMNs along the venular endothelium was unaffected. Thus, these experiments confirm and extend earlier *in vitro* findings of the critical role of the membrane glycoprotein complex, CD18, in PMN adherence and chemotaxis.

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THE MEMBRANE glycoprotein complex designated CD18 (LFA-1/Mac-1)¹ is critical for the *in vitro* adherence of stimulated polymorphonuclear leukocytes (PMNs) to endothelial cells.^{2,3} CD18-deficient patients have recurrent infections without pus formation. It has been postulated that the absence of the membrane-associated glycoprotein(s) in these patients prevents adherence of PMNs to the endothelium following activation of the former cell and accounts for the failure of their PMNs to migrate to an inflammatory locus.^{2,3} The CDw18 membrane glycoprotein is recognized by the murine monoclonal antibody (MoAb) 60.3.⁴ Addition of MoAb 60.3 to normal PMNs induces *in vitro* defects in PMN spreading, adherence, and chemotaxis similar to those observed in the patients.⁵

The present study was undertaken to investigate whether MoAb 60.3 also would reduce PMN accumulation *in vivo*, using rabbit inflammatory skin lesions and the rabbit tenuissimus muscle prepared for intravital microscopy. The rabbit was selected for these studies because the antigen recognized by MoAb 60.3 was present in high density on rabbit peripheral blood PMNs when analyzed by FACS and because MoAb 60.3 inhibited stimulated rabbit PMN adherence to cultured endothelial cells (J. Harlan, unpublished observations, 1986).

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MATERIALS AND METHODS

Inflammatory skin lesions. Male New Zealand White rabbits, weighing between 1.2 and 1.5 kg, their backs preshaved 12 hours prior to the experiment, were used. The animals were anesthetized with diazepam 5 mg/kg intramuscularly (IM) and thereafter with 10 to 20 mg/kg intravenously (IV) methohexital sodium. The rabbits were either treated with 500 µl/kg of saline IV control (n = 6) or 2 mg/kg of MoAb 60.3 IV (n = 6). Ten minutes later, 10 µCi of ¹²⁵I-labeled human serum albumin was given IV as a plasma tracer, and 100 µL of saline, fMLF (10⁻⁵ mol/L), porcine C5a (2.8 × 10⁻⁷ mol/L and 2.8 × 10⁻⁶ mol/L) and histamine (8 × 10⁻⁴ mol/L) were randomly injected intradermally in duplicate sites in the dorsal region of the animals. Four hours later, the rabbits were reanesthetized, a blood sample was drawn, the rabbits were killed by an overdose of anesthesia, and the skin lesions were carefully removed and dissected free of fat and muscle tissue. After radioactivity measurements of tissue and plasma samples were made, the skin lesions were ground in 0.5% hexadecyltrimethylammonium bromide, freeze-thawed three times, and the myeloperoxidase (MPO) activity was measured in the supernatants. The MPO content of the skin lesions is expressed in units of MPO for each gram of wet weight and albumin extravasation as microliters of plasma exuded for each gram of wet weight.⁶

Intravital microscopy. Direct observation of PMN adherence to the microvascular endothelium was performed using the rabbit tenuissimus muscle prepared for intravital microscopy.^{7,8} The animals were anesthetized with diazepam (5 mg/kg, IM) and thereafter with 20% urethane (1.5 g/kg, IV). LT_{B₄} (final concentration 10⁻⁶ mol/L) and zymosan-activated rabbit serum (ZAS; diluted 1:20) were applied topically to the muscle for a period of 20 minutes. Complement activation following a zymosan concentration of 20 mg/mL of serum, as used here, results in ~3 µg of C5a_{desArg}/mL serum.⁹

Statistics. Wilcoxon signed-rank test was used for calculating *P* values. A *P* value <.05 was considered significant and is denoted in Figs 1 and 2 with an asterisk. Data are given as mean ± SEM.

RESULTS

MPO has previously been shown to be a useful and reliable marker enzyme for PMN accumulation *in vivo*,⁶ and was therefore used to assess PMN accumulation in the inflammatory skin lesions in this study. As shown in Fig 1, in the control animals the chemotactic agents (fMLF, LT_{B₄}, and

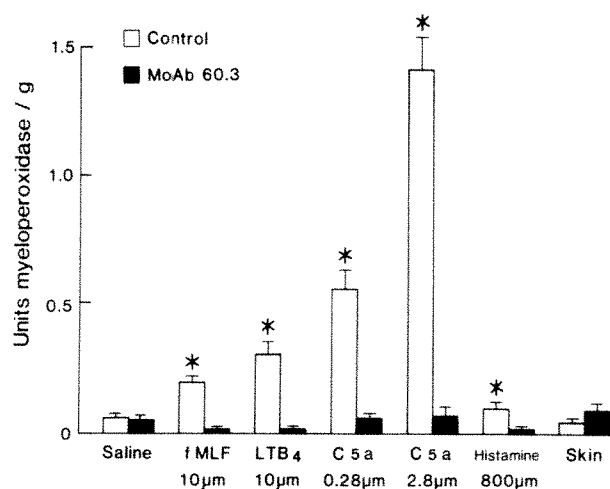


Fig 1. PMN accumulation, as measured by myeloperoxidase, in inflammatory skin lesions from the dorsal region of the rabbit. The animals were pretreated with either saline (control) or MoAb 60.3 (2 mg/kg) intravenously 10 minutes prior to the experiment. Values represent mean $\pm$ SEM of six experiments. Asterisks indicate a significant difference as compared with saline-injected skin sites.

C5a) caused a marked local PMN accumulation in the skin lesions as compared with the saline-injected skin lesion. On a molar basis, porcine C5a was found to be a more potent chemotactic agent than both fMLF and LTB₄, although all three agents caused a significant increase in PMN accumulation over saline-injected skin sites in control animals. Moreover, histamine caused a small but significant increase in tissue MPO content. In rabbits pretreated with MoAb 60.3, however, PMN accumulation was completely absent and comparable to the saline lesions as well as intact uninjected skin. Extravasation of PMNs at chemotaxin-injected sites is followed by plasma leakage.¹⁰⁻¹² Plasma leakage in the skin lesions is shown in Fig 2. All the chemotactic agents (fMLF, LTB₄ and C5a), as well as histamine, caused a significant increase in albumin extravasation as compared with the saline-injected site in the control animals. In MoAb 60.3-treated animals, no increase in plasma leakage could be observed for the chemotactic agents, whereas histamine still caused a significant plasma leakage in these rabbits.

In three experiments, the Fc-portion of MoAb 60.3 was removed by pepsin cleavage and the F(ab')₂ portion was used. These fragments inhibited PMN accumulation and PMN-dependent plasma leakage to the same extent as the whole IgG molecule (data not shown).

Direct observation of the tenuissimus muscle after topical application of LTB₄ or ZAS in control animals induced vigorous PMN adherence to the endothelium of the post-capillary venules and subsequent extravasation into the interstitial space. This is shown for ZAS application in Fig 3A and B. In contrast, PMN adherence and extravasation was not observed following LTB₄ or ZAS application in MoAb 60.3-treated rabbits. Figure 3C and D show a post-capillary venule in the tenuissimus muscle of a MoAb

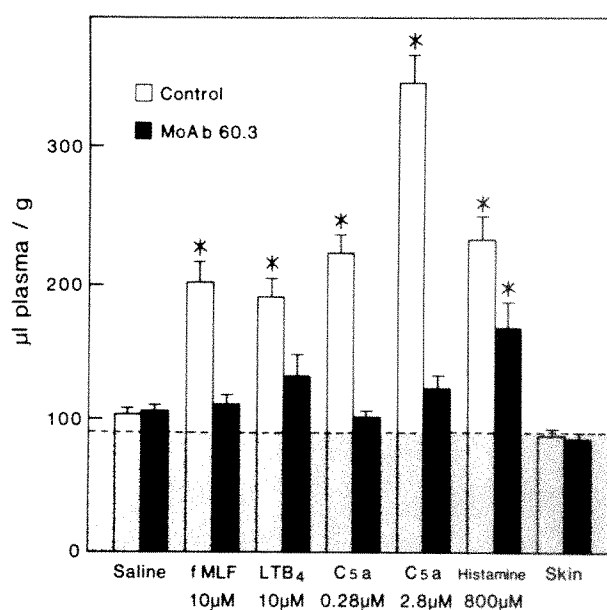


Fig 2. Plasma leakage, as measured by ¹²⁵I-human serum albumin extravasation in inflammatory skin lesions from the dorsal region of the rabbit. The animals were pretreated with either saline (control) or MoAb 60.3 (2 mg/kg) intravenously 10 minutes prior to the experiment. Values represent mean $\pm$ SEM of six experiments. Asterisks indicate a significant difference as compared with saline-injected skin. Shaded area represents normal skin plasma volume.

60.3-treated rabbit before and after ZAS application. Rolling of the leukocytes along the venular endothelium was not reduced by MoAb 60.3, but rather was slightly enhanced in these animals. The striking inhibition of PMN adherence and extravasation by MoAb 60.3 lasted throughout the experiment, ie, for up to 5 hours.

DISCUSSION

We demonstrated that MoAb 60.3 prevents PMN accumulation in vivo, using two different in vivo models in the rabbit; the inhibition of PMN accumulation by MoAb 60.3 is in accordance with, and extends, earlier in vitro findings in which MoAb 60.3 inhibited adherence of stimulated human PMNs to human endothelial cell monolayers.²⁻⁵ Intravital microscopy demonstrated PMN sticking to the postcapillary venular wall, and subsequent extravasation was completely absent in animals pretreated with MoAb 60.3, supporting the results obtained from the inflammatory skin lesions. That rolling of leukocytes along the venular endothelium was not affected by MoAb 60.3 suggests a different adherence mechanism for the rolling and sticking of leukocytes, or, alternatively, the involvement of two different cell populations.

Previous in vitro studies demonstrated that by preventing close approximation of the neutrophil and the endothelial cells, MoAb 60.3 reduced PMN-mediated endothelial injury.¹³ Inhibition of PMN adherence and/or extravasation by MoAb 60.3 also abolished PMN-dependent plasma leak-

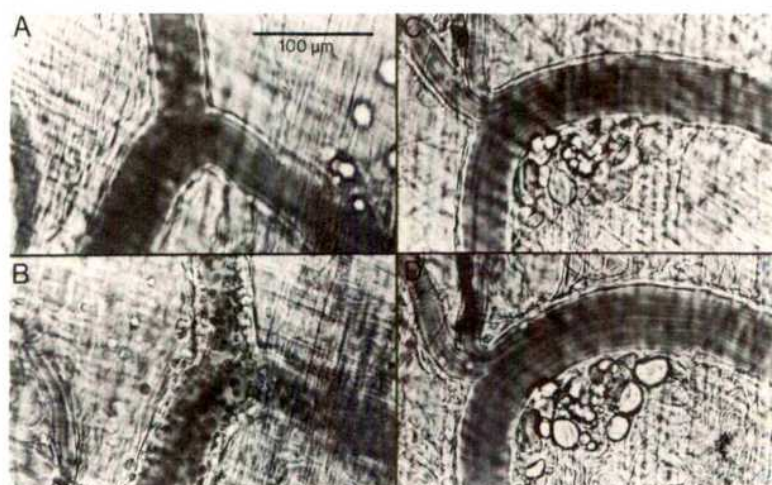


Fig 3. The upper left panel (A) shows a postcapillary venule in the rabbit tenuissimus muscle prepared for intravital microscopy (control animal). The lower left panel (B) shows the same venule 1 hour after topical application of zymosan-activated serum (ZAS) with abundant adherent and extravasated PMNs. The upper right panel (C) shows a postcapillary venule in a rabbit pretreated with MoAb 60.3 (2 mg/kg). The lower right panel (D) shows the same venule after a 20-minute topical application of ZAS. In contrast to panel B, no adherent or extravasated PMNs are observed.

age, whereas the PMN-independent plasma leakage was unaffected by MoAb 60.3 treatment.

We conclude, confirming earlier *in vitro* findings, that the membrane glycoprotein complex CD18 is indeed critical for PMN adherence and extravasation *in vivo*. Moreover, MoAb 60.3 reduces PMN-dependent plasma leakage in the inflammatory skin lesions. These findings make MoAb 60.3 a powerful tool in studying the role of PMNs in various

inflammatory events as well as in controlling the inflammatory process.

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CONCISE REPORT

The Cellular Basis for Different Fetal Hemoglobin Levels Among Sick Cell Individuals With Two, Three, and Four Alpha-Globin Genes

By G.J. Dover, V.T. Chang, S.H. Boyer, G.R. Serjeant, S. Antonarakis, and D.R. Higgs

Fetal hemoglobin (HbF) levels vary widely among individuals with sickle cell anemia (SS). Previous studies have suggested that HbF levels in SS individuals with alpha-thalassemia (two or three functional alpha-globin genes) are lower than HbF levels in SS individuals with four normal alpha-globin genes. Using immunocytochemical techniques, we studied F cell production as measured by % F reticulocytes, the amount of HbF per F cell, and the preferential survival of F cells versus non-F cells in 51 subjects with four alpha genes, 32 subjects with three alpha genes, and 18 subjects with two alpha genes. Comparison between alpha-globin gene groups was performed for the total sample as well as for a subset of 82 individuals who had replicate samples and a further subset of 39 age-matched individuals. %HbF levels were 6.8, 4.9, and 4.5 percent for the total four-, three-, and two-alpha-globin-gene groups, respectively. The percentage of F

reticulocytes, percentage HbF per F cell, and the enrichment ratio (% F cell/% F reticulocytes) did not change significantly with alpha-globin gene number. Moreover, no correlation existed between alpha-globin gene number and the absolute number of F cells in any group studied. However, there was a strong inverse correlation ($r = -0.407$, $P = .0001$) between non-F cell levels ($1.7 \pm 2.2 \pm 5$, $3.0 \pm 1.0 \times 10^{12}/L$) and decreasing alpha-globin gene number. These data suggest that falling HbF levels among SS individuals with lessened numbers of alpha-globin genes reflect prolonged survival of non-F cells and are not due to intrinsic differences in F cell production or in the amount of HbF per F cell. The improved survival of non-F cells in SS alpha-thalassemia is presumed to be due to the lower MCHC observed in such individuals.

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ALPHA-thalassemia modifies many hematologic parameters in individuals who have homozygous sickle cell disease (SS). SS individuals with only two alpha-globin genes have higher hemoglobin levels, lower reticulocyte counts, and lower mean corpuscular hemoglobin concentrations (MCHC) than SS individuals with four alpha-globin genes.¹⁻³ In the largest study to date,³ fetal hemoglobin (HbF) levels were lower in SS individuals with two alpha-globin genes than in SS individuals with four alpha-globin genes. The reason for this difference in HbF levels among SS patients has been obscure.

In normal and SS individuals HbF is confined to a subset of erythrocytes called F cells.⁴ HbF levels in patients with SS disease is the summation of three independent variables; first, the number of F cells produced, as estimated by the percentage of F reticulocytes; second, the amount of HbF per F cell; and third, the preferential survival of F cells versus those red cells which contain no detectable HbF (non-F cells).⁵ In a previous study utilizing a small number of SS individuals (10 to 15 subjects per group) we saw no difference in the percent HbF, percentage of F reticulocytes, or the percent HbF per F cell between SS individuals with two or four alpha-globin genes.⁶ Now, in analysis of 101 SS individuals, we substantiate the finding of others³ that HbF levels are lower in SS individuals with two alpha-globin genes than in those with four alpha-globin genes. Furthermore, in this report, we analyze in detail the three cellular variables that determine HbF levels in order to determine the basis for different levels of HbF in these individuals. We conclude that F cell production and the percent of HbF per F cell are the same in SS patients with and without alpha-thalassemia. We find that the difference in HbF levels is directly attributable to the enhanced survival of the non-F cells in SS individuals with coexisting alpha-thalassemia.

MATERIALS AND METHODS

All patients evaluated attended the Medical Research Council Sickle Cell Clinic in Kingston, Jamaica. Eighty-three SS individuals

(51 with four alpha-globin genes, 32 with three alpha-globin genes) were obtained from a previous study of SS siblings randomly selected from the clinic registry.⁷ These patients had not been transfused and had no recent infection or crisis. Alpha-globin gene number was not known at the time of their selection. Eighteen two-alpha-globin gene SS individuals were randomly selected from clinic patients previously identified as having two alpha-globin genes in a survey of the same clinic population.² The average age ($\pm$ SD) for the entire population was 20.6 ± 9.5 years, with no significant age differences between the alpha-globin gene groups. Informed consent was obtained in accordance with the guidelines of both the Johns Hopkins University Medical School Joint Committee of Clinical Investigation and the Clinical Investigation Committee of the University of West Indies.

To ensure reproducibility of hematologic parameters, a second sample was obtained within a one-year interval from most subjects. Eighty-two persons underwent such re-examination. In addition, an age-matched subset was analyzed retrospectively including 13 age-matched individuals from each alpha-globin gene number. Statistical analysis was performed for the total population, for subjects in which second samples had been drawn, and for the age-matched group.

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All samples of blood were obtained from venous blood collected in EDTA. Peripheral blood reticulocyte counts were obtained using conventional new methylene blue staining, and red cell indices were performed using an electronic cell counter (Coulter ZBI 6, Coulter Electronics, Hialeah, FL). Alpha-globin gene number was determined or redetermined, in cases of two-alpha-globin gene subjects, by DNA restriction endonuclease analysis utilizing DNA obtained from peripheral blood leukocytes.⁸ Percentage of F reticulocytes was assayed by radial immunoprecipitate reactions.⁹ F cell counts were performed either by the above procedure or by analysis of fixed red cells with a monoclonal anti-HbF antibody.¹⁰ Percentage of HbF levels was determined by alkaline denaturation techniques.¹¹

Statistical distributions were tested for normality by a chi-square test, based on the observed mean and variance. Bartlett's test was used to test for equality of variance. For values that were not normally distributed (% HbF, % reticulocytes), logarithmic transformation was used. The degree of linear correlation between alpha-globin gene numbers and hematologic parameters were assessed by linear correlation coefficient (*r*). Where the distributions were nonnormal, or the variances were unequal, the Spearman-Rank correlation test was used.

RESULTS

Table 1 summarizes the hematologic data for the overall group. No significant correlations were found between alpha-globin gene number and F enrichment ratios, F reticulocytes, or the percentage of HbF per F cell. The remaining hematologic parameters were highly correlated with alpha-globin gene groups: Picograms of HbF per F cell, percentage of F cells, percentage of HbF, MCHC, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and percentage of reticulocytes; all decreased significantly with decreasing alpha-globin gene number. In contrast, hemoglobin levels and red cell number increased with decreasing alpha-globin gene numbers.

Table 2 provides a comparison of the absolute numbers of F cells and non-F cells in SS patients partitioned according to alpha-globin gene number. Although the percentage of F reticulocytes (Table 1) are not correlated with alpha-globin gene number, the absolute numbers of F reticulocytes (Table 2) significantly decrease as the alpha-globin gene number falls. This decrease is due to the significant decrease in the total reticulocyte percentages noted in Table 1. Non-F reticulocyte levels seem to fall with decreasing alpha-globin genes but not significantly so. Although absolute F cell numbers were not correlated with alpha-globin gene numbers, the absolute number of non-F cells per liter was negatively correlated ($r = -0.407$, $P = .0001$) with decreasing alpha-globin gene. The upshot is that alpha-thalassemia is associated with prolonged survival of non-F cells. The outcome is the same when analysis like that in Table 2 is applied to either mean values from 82 subjects who had repeat tests or to 39 age-matched subjects.

DISCUSSION

The intent of this paper was to analyze the origins of differences in HbF levels among individuals with SS disease who have different numbers of alpha-globin genes. Like some previous investigators,^{2,12} we found a significant inverse correlation between the percentages of HbF and the number of alpha-globin genes. In order to determine the origins of these differences, we considered the fact that HbF is discontinuously distributed among red cells in these individuals.⁵ We analyzed the three major variables that contribute to the percentage of HbF levels found in peripheral blood. The first of these, ie, the percentage of F reticulocytes, was not different among the three alpha-globin groups studied (Ta-

Table 1. Hematologic Features in SS Individuals and Varying Numbers of Alpha-Globin Gene (Mean $\pm$ SD)

	Alpha-Globin Genes			Statistics	
	4 N = 51	3 N = 32	2 N = 18	r	P
F enrichment ratio*	1.75 $\pm$.89	1.58 $\pm$.90	1.47 $\pm$.55	.130	NS
F reticulocyte (percent)	20.9 $\pm$ 10.5	19.7 $\pm$ 11.3	16.5 $\pm$ 7.9	.149	NS
F cell (percent)	34.4 $\pm$ 17.0	26.5 $\pm$ 14.6	24.0 $\pm$ 15.3	.262	.01
HbF/F cell (pg)†	7.1 $\pm$ 2.2	6.1 $\pm$ 1.8	5.5 $\pm$ 2.7	.403	.0001
HbF/F cell (%)‡	22.2 $\pm$ 6.4	21.9 $\pm$ 6.2	23.2 $\pm$ 12.2	.167	NS
Hemoglobin F§ (percent)	.89(6.8) $\pm$.28	.77(4.9) $\pm$.28	.73(4.3) $\pm$.24	.242	.02
Hemoglobin	8.2 $\pm$ 1.0	8.4 $\pm$ 1.4	9.2 $\pm$ 1.6	-.241	.02
MCHC (g/dL)	34.1 $\pm$ 4.5	33.3 $\pm$ 2.6	32.1 $\pm$ 1.7	.304	.005
Red cells ($\times 10^{12}$ /L)	2.63 $\pm$.39	3.04 $\pm$.60	3.91 $\pm$.82	-.627	.001
MCV (fl)	91.6 $\pm$ 6.9	82.5 $\pm$ 6.8	72.7 $\pm$ 5.5	.725	.0001
MCH (pg)	31.6 $\pm$ 2.9	28.0 $\pm$ 3.0	23.7 $\pm$ 2.1	.716	.0001
Reticulocytes§ (percent)	1.03(9.6) $\pm$.21	.96(8.1) $\pm$.19	.83(5.8) $\pm$.19	.316	.002

*F enrichment ratio calculated as the (F cell percent)/(F reticulocyte percent).

†Picograms (pg) of HbF/F cell = (MCH $\times$ %HbF)/F cell%.

‡HbF/F cell (%) = [pgHbF/F cell/MCH] $\times$ 100. Note MCH of F cell and non-F cells are the same.¹⁰

§These values were calculated after logarithmic transformation. For hemoglobin F values, log(%HbF + 1); for reticulocytes, log(%reticulocytes + 1).

Figures in parentheses represent means reexpressed in original units.

||r = regression coefficient; P = probability of hematologic variable correlated with alpha-globin gene number (see Methods); NS = not significant (>0.05).

Table 2. Absolute Number of F Cells and Non-F Cells in SS Individuals and Varying Numbers of Alpha-Globin Gene Loci (Mean $\pm$ SD)

	Alpha-Globin Genes			Statistics	
	4	3	2	r	P
F reticulocytes* ($\times 10^{12}/L$)	.056 $\pm$.032	.051 $\pm$.035	.043 $\pm$.030	.231	.023
F cells ($\times 10^{12}/L$)†	.93 $\pm$.50	.84 $\pm$.57	.98 $\pm$.57	.129	NS
Non-F reticulocytes* ($\times 10^{12}/L$)	.23 $\pm$.13	.21 $\pm$.09	.20 $\pm$.12	.106	NS
Non-F cells ($\times 10^{12}/L$)†	1.70 $\pm$.17	2.20 $\pm$.49	2.95 $\pm$ 1.0	-.407	.0001

*Absolute number of F or non-F reticulocytes = (RBC) $\times$ (reticulocyte percent) $\times$ (percentage of F or non-F reticulocytes).

†Absolute number of F or non-F cells = (RBC) $\times$ (percentage of F cells or non-F cells).

ble 1). However, the absolute number of F reticulocytes decreased significantly with falling alpha-globin gene number, the level in two-alpha-globin-gene individuals being 77% of that in four-alpha-globin-gene individuals (Table 2). In the absence of significant between-group differences in the percentage of F reticulocytes (Table 1), we presume that the fall in their absolute number is due to a concomitant and significant decrease in overall reticulocyte production (Table 1). These changes would not be expected to have an appreciable effect on HbF levels, since F reticulocytes, in themselves, represent only a few percent of all HbF-bearing cells. While there were significant differences in the second variable affecting HbF production, ie, picograms of HbF per F cell, among the alpha-globin gene categories, there was a parallel fall in the MCH of F cells. Thus no change developed in the actual percentage of HbF per F cell.

The third variable that affects HbF levels is the difference in survival of F cells versus non-F cells in the peripheral blood. This difference in preferential survival was measured two ways. As assessed by comparison of enrichment ratios (% F cells/% F reticulocytes), a decline in enrichment ratios with decreasing alpha-globin gene number (Table 1) was observed. However, the magnitude of this change was not statistically significant. The difficulty with such analysis of enrichment ratios is that it is perturbable by known variation in each of the two estimates, F reticulocytes and F cells, that contribute to it. Consequently, such estimates are less precise than the measure of absolute numbers of non-F cells which utilize only one HbF-centered variable. Here, a highly significant inverse correlation in the number of non-F cells per liter between groups of individuals with decreasing alpha-globin gene number was observed (Table 2). Since non-F reticulocytes did not change between groups, the higher concentration of non-F cells in the two- and three-alpha-globin-gene groups indicate that non-F cells survive

longer in SS individuals with alpha-thalassemia in comparison to SS individuals with four alpha-globin genes.

Why do non-F cells last longer in SS individuals with alpha-thalassemia? We suppose that it is the indirect outcome of a lessened MCHC associated with alpha-thalassemia (Table 1).^{1-3,6} Sick cell hemoglobin (HbS) polymerization is highly dependent upon HbS concentration within the red cell.¹⁴ Recently, Brittenham demonstrated that intracellular HbS polymer levels, calculated on the basis of MCHC and HbS concentration, accounted for at least 80% of the variation in hemoglobin levels among different SS syndromes.¹⁵ Alpha-thalassemia leads to decreased MCHC in all cells⁶ and to a decreased proportion of dense irreversibly sickled cells.^{6,16} The dense cell fraction (MCHC > 37 g/dL) has two hallmarks: it contains very few F cells and exhibits shorter survival than less dense cells.⁶ A decrease in the proportion of dense cells should therefore lead to a relative decrease in the difference between F cell survival and non-F cell survival. The fact is that the majority of non-F cells in SS individuals with two alpha-globin genes have MCHC that are near normal.⁶ Consequently, in this setting, one would expect that non-F cells with near-normal MCHC would last longer than they do when MCHC is elevated. Thus, in these respects, our data indicating prolonged survival of non-F cells in SS individuals with two and three alpha-globin genes are consistent with previous reports.

In summary, differences in HbF levels among SS individuals with different numbers of alpha-globin genes are not due to differences in F cell production or percentage of HbF per F cell. The difference is due to the relatively prolonged survival of non-F cells in alpha-thalassemic SS individuals compared to non-F cells in SS individuals with four alpha genes. We speculate that previously demonstrated declines in MCHC among SS alpha-thalassemic individuals account for this prolonged survival.

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Amantadine Potentiates T Lymphocyte Killing by an Anti-Pan-T Cell (CD5) Ricin A-Chain Immunotoxin

By Salvatore Siena, Silvia Villa, Marco Bregni, Gianni Bonnadonna, and A. Massimo Gianni

The studies described in this report demonstrate that 1-adamantanamine hydrochloride (amantadine) is a potent enhancer of the cytotoxic activity of the anti-pan-T lymphocyte (CD5) T101 monoclonal antibody conjugated to purified ricin A-chain (T101-immunotoxin; T101-IT). We also demonstrate that T101-IT in the presence of amantadine does not induce immunotoxin-mediated cytotoxicity in nontarget cells such as human marrow hematopoietic

progenitor cells. These results provide further knowledge for the improvement of ex vivo purification of human bone marrow from normal or leukemic T cells prior to allogeneic or autologous stem cell transplantation, respectively. Furthermore, since amantadine has long been employed safely in human therapy, its use in conjunction with immunotoxins might be exploited in vivo.

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CELL-specific antibodies coupled to the A-chain subunit of ricin, abrin, or diphtheria toxin give rise to hybrid molecules, referred to as A-chain immunotoxins, that possess specific cytotoxicity against target cells.^{1,2} Unfortunately, A chain immunotoxins do have limitations, particularly in terms of potency and slow kinetics of cytotoxicity. These problems have been partly obviated in vitro by long incubations under conditions nonreproducible in vivo.¹⁻⁸

In a systematic search for substances of potential clinical activity capable of improving A-chain translocation into the cytosol, we tested the symmetrical primary amine amantadine. This drug is selectively concentrated in lysosomes and other acidic organelles,⁹ is capable of elevating endosomal and lysosomal pH,¹⁰ and interferes with receptor recycling¹¹ at the level of compartment of uncoupling receptor and ligand.¹²

We report here that in vitro amantadine, at concentrations nontoxic to human hematopoietic progenitor cells, enhances the activity and accelerates the kinetics of the anti-pan-T cell (CD5) T101 ricin A-chain immunotoxin (T101-IT) against normal T lymphocytes.

MATERIALS AND METHODS

T101 ricin A chain immunotoxin (T101-IT; SR 41322; batch RBM07) consists of the murine monoclonal IgG_{2a} antibody T101 (Hybritech, San Diego, Calif) chemically bound to purified ricin A chain.^{1,5,7} T101 antibody recognizes the 65 kD antigen present on human peripheral blood T lymphocytes (CD5[T, 65 kD] T101).¹³

Normal bone marrow cells were obtained by aspiration from the iliac crests of volunteers, who gave informed consent in accordance with institutional guidelines. Peripheral blood (PB) and bone marrow (BM) mononuclear cells were prepared by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Collected cells were washed 3 times with Hanks' buffered saline solution (HBSS) before treatment with T101-IT. Ficoll-Hypaque-isolated mononuclear cells at 10⁷/mL (PB) or 2 × 10⁷/mL (BM) were suspended in HBSS containing amantadine hydrochloride (1-Adamantanamine HCl, Sigma, St. Louis, Mo) and/or ammonium chloride. The incubation medium was adjusted to pH 7.8 by addition of 2 N sodium hydroxide and contained 2% fetal calf serum (FCS) and 10 mmol/L HEPES buffer. After ten minutes at 37 °C, incubation with T101-IT was initiated in ice for ten minutes and then continued at 37 °C. At the end of incubation, cells were washed 4 times at 4 °C with 1% FCS-HBSS and assayed according to the experimental protocol.

To measure lectin-induced polyclonal T cell proliferation, PB mononuclear cells were stimulated with 10 µg/mL phytohemagglutinin (PHA-P, Difco, Detroit, Mich). Mononuclear cells (10⁵ cells/well) were cultured in round-bottomed microtiter plates in 0.2 mL

RPMI 1640 supplemented with 15% FCS, 10 mmol/L HEPES buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. Triplicate cultures were incubated at 37 °C in a 5% CO₂ humidified atmosphere for 72 hours and then labeled with 1 µCi/well tritiated thymidine (³H-TdR). After eight hours, ³H-TdR incorporation was measured by standard scintillation counting techniques.

To measure lymphocyte proliferative response to allogeneic cells in mixed lymphocyte culture (MLC), responder PB mononuclear cells (5 × 10⁴) were cultured with 5 × 10⁴ irradiated (2,500 rad) stimulator cells (1:1 mixture of allogeneic PB mononuclear cells from two unrelated individuals). Triplicate cultures were performed in supplemented RPMI 1640 with 15% FCS in round-bottomed microtiter plates at 37 °C in a 5% CO₂ humidified atmosphere. After 120 hours, plates were pulsed with ³H-TdR 1 µCi/well. After an additional 24 hours, ³H-TdR incorporation was measured by standard scintillation counting techniques.

The frequency of clonable T lymphocytes in BM samples was evaluated by a limiting dilution microculture assay in PHA-IL 2 feeder cell containing medium according to Kernan et al¹⁴ with minor modifications as previously described.⁸ T lymphocyte frequency was determined by the minimum chi-square method from the Poisson distribution relationship between the cell number seeded per well and the logarithm of the percentage of negative culture wells.¹⁵

The frequency of multipotential (CFU-GEMM), erythroid (BFU-E), and granulomonocytic (CFU-GM) hematopoietic progenitor cells was evaluated by clonal assays as previously described.¹⁶

RESULTS

Unless otherwise specified, 10 nmol/L T101-IT was employed throughout the experiments presented here. In previous studies^{1,7,8} this concentration was found to be optimal in terms of high immunotoxin activity and lack of cytotoxicity to human hematopoietic progenitors.

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Viability of PB as well as BM mononuclear cells was assessed by Trypan blue dye exclusion test at the end of four hours incubation. Treatment with 0.1 to 1.5 mmol/L amantadine and T101-IT did not affect cell viability. Higher concentrations, ie, 1.75 and 2 mmol/L amantadine either with or without T101-IT, reduced the number of total viable cells to $84.75\% \pm 13.36\%$ and $27.45\% \pm 9.68\%$ of medium controls, respectively.

In the absence of enhancer molecules, four-hour treatment with T101-IT did not change the mitogenic response of PB T lymphocytes. In contrast, the addition of amantadine to the incubation medium enhanced the activity of T101-IT in a dose-related manner (Fig 1). The most effective nontoxic concentration was 1 mmol/L amantadine which in the presence of 10 nmol/L T101-IT resulted in the inhibition of T lymphocyte mitogenic response to $2.18\% \pm 2.50\%$ of medium controls ($n = 4$). The same amantadine concentration potentiated also the T101-IT inhibition of PB T cell response to allogeneic cells in MLC ($11.72\% \pm 1.26\%$, $12.23\% \pm 1.32\%$, and 12.98 ± 1.40 of medium, amantadine, and T101-IT controls, respectively; $n = 1$).

In parallel experiments, T101-IT in the presence of 10 mmol/L ammonium chloride reduced the PB T cell proliferative response to PHA and to allogeneic cells in MLC to only $41.23\% \pm 21.60\%$ ($n = 3$) and $32.5\% \pm 5.38\%$ ($n = 1$) of controls, respectively. T101-IT in the presence of 20 mmol/L ammonium chloride, or 10 mmol/L methylamine, or 100 μ mol/L chloroquine reduced the T cell proliferative response to PHA to $20.04\% \pm 9.70\%$, $42.30\% \pm 11.30\%$, and $49.00\% \pm 9.83\%$ ($n = 2$) of controls, respectively.

The kinetics of the inhibition of PB T lymphocyte proliferative response to PHA by T101-IT in the presence of 1 mmol/L amantadine or 20 mmol/L ammonium chloride, is shown in Fig 2. Amantadine sped the rate at which T101-IT inhibited T lymphocyte proliferation more dramatically than did ammonium chloride. In addition, the overall enhancement of T101-IT activity was markedly higher with amantadine than with ammonium chloride. A two-hour incubation

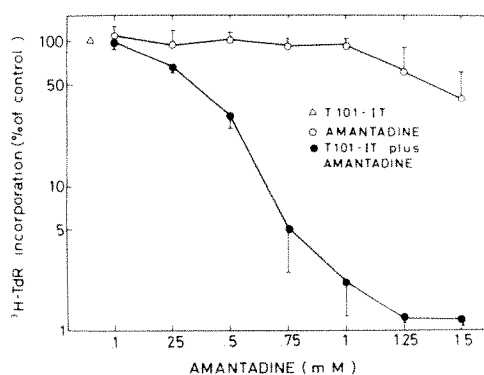


Fig 1. Effects of amantadine and/or T101-IT on lymphocyte proliferative response to phytohemagglutinin. Peripheral blood mononuclear cells (10^7 /mL) were treated with 10 nmol/L T101-IT and/or various concentrations of amantadine before culturing in the presence of PHA. Cultures were labeled at 72 hours with ^3H -TdR, and the results are presented as the percentage of thymidine incorporation in treated samples relative to untreated controls. Amantadine potentiates the T101-induced inhibition of mitogenic T cell proliferative response in a dose-related manner.

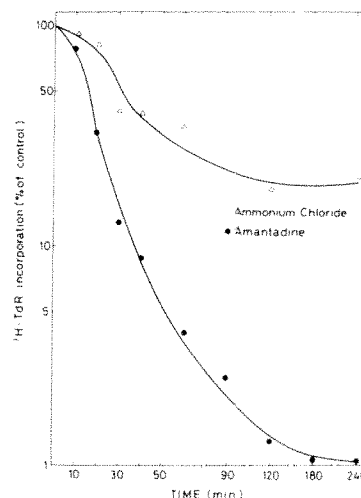


Fig 2. Effect of T101-IT in the presence of amantadine or ammonium chloride on the rate of T cell mitogenic proliferative response inhibition. Peripheral blood mononuclear cells (10^7 /mL) were treated with 10 nmol/L T101-IT and 1 mmol/L amantadine or 20 mmol/L ammonium chloride. At the times indicated, the cells were washed 4 times at 4°C and then cultured in the presence of PHA. Cultures were labeled at 72 hours with ^3H -TdR, and the results are presented as the percentage of thymidine incorporation in treated samples relative to untreated controls.

with T101-IT and 1 mmol/L amantadine was sufficient to inhibit lymphocyte proliferative response to $1.29\% \pm 0.41\%$ ($n = 2$) of control medium.

To address the possibility that amantadine is toxic to an accessory cell(s) necessary for the *in vitro* T cell activation and proliferation, PB mononuclear cells treated with T101-IT and amantadine were mixed at different ratios with autologous medium-treated PB mononuclear cells and assayed for PHA proliferative response. The observed rate of ^3H -TdR incorporation corresponded to the expected values calculated on the basis of dilution in the cell mixtures, thus ruling out a toxic effect of amantadine plus T101-IT on an accessory cell(s) (data not shown).

Based on the results of the experiments in which PB lymphocytes were the target of the immunotoxin cytotoxicity, human BM mononuclear cells at 20×10^6 /mL were incubated for two hours with T101-IT alone or T101-IT plus either amantadine or ammonium chloride. The frequency of BM T lymphocytes was evaluated by a limiting dilution microculture assay which is the most sensitive method for the detection and enumeration of very low numbers of residual BM T cells.^{14,17}

In the presence of 1 mmol/L amantadine, a two-log depletion of BM T lymphocytes was achieved. In contrast, when 10 mmol/L ammonium chloride was employed instead of amantadine, only one log reduction of BM T lymphocytes was achieved. In all instances, the treatment with T101-IT and/or either enhancer molecule did not significantly alter the recovery of CFU-GEMM, BFU-E, and CFU-GM hematopoietic progenitor cells (Table 1).

DISCUSSION

The data presented in this report demonstrate that amantadine enhances T101-IT cytotoxic effect and that it is

Table 1. Specific Depletion of Human Bone Marrow T Lymphocyte by T101-IT in the Presence of Amantadine or Ammonium Chloride

	Total Nucleated Cells	T Cells	Percentage of Medium-Treated Control		
			Hematopoietic Progenitor Cells		
			CFU-GEMM	BFU-E	CFU-GM
T101-IT	87.1 ± 10.3	101.1 ± 9.8	ND	ND	92.0 ± 7.9
T101-IT plus Amantadine	89.4 ± 8.9	2.20 ± 0.38	72.0 ± 22	69.9 ± 4.1	193.7 ± 75.3
T101-IT plus Ammonium Chloride	91.5 ± 15	13.12 ± 2.10	69.0 ± 19	73.9 ± 15.3	97.1 ± 25.1

Bone marrow mononuclear cells at $20 \times 10^6/\text{mL}$ were incubated for two hours with 10 nmol/L T101-IT in the presence of either 1 mmol/L amantadine or 10 mmol/L ammonium chloride. After 4 washings, cells were assayed for the number of total viable cells, clonable T cells, and hematopoietic progenitor cells. Values shown are the mean $\pm$ SD from two separate experiments. Control CFU-GEMM, $13.6 \pm 3.4/5 \times 10^4$; BFU-E, $336.4 \pm 64.5/5 \times 10^4$; CFU-GM, $88 \pm 51.1/10^5$.

superior to ammonium chloride both in terms of rapidity of action and level of T101-IT cytotoxicity against PB T lymphocytes. In 1984, Casellas et al reported that the degree of enhancement of T101-IT cytotoxicity against T leukemia CEM cells by 1 mmol/L amantadine is inferior to 100 $\mu\text{mol/L}$ chloroquine, 10 mmol/L ammonium chloride, or 10 mmol/L methylamine.⁵ The same authors chose ammonium chloride in their subsequent work.⁷ The present study shows that when targets of T101-IT cytotoxicity are normal T cells, the most effective potentiator is amantadine followed by ammonium chloride, methylamine, and chloroquine. The diversity of our results compared to those of Casellas et al supports the notion that A-chain immunotoxins exhibit variability in target cell toxicity.²

The immediate application of these data is the use of amantadine as optimal potentiator of T101-IT for ex vivo depletion of mature T lymphocytes from bone marrow grafts prior to allogeneic transplantation. Recently, Kernan et al have shown that the number of clonable residual T lymphocytes measured by a limiting dilution assay¹⁴ correlates with the development of GVHD in 32 leukemic patients following transplantation of histocompatible donor T cell-depleted BM. In particular, these authors found that 24 patients who received a median of 3.72×10^4 (0.92 to 16.0×10^4) residual BM T cells per kg of recipient body weight did not develop GVHD. Of the 8 patients who received a median of 23.99×10^4 (18.63 to 43.93×10^4) T cells per kg, 4 had no GVHD, and 4 developed grade I-II (skin only) GVHD.¹⁸ Employing

the same limiting dilution assay for quantitation of residual BM T cells, we show in this report that T101-IT in the presence of amantadine induces a 2 log reduction of BM T lymphocytes without adversely affecting multipotential hematopoietic progenitor cells. Typical untreated BM grafts contain 1.0×10^9 (0.7 to 1.4×10^9) clonable T lymphocytes.¹⁴ Following a 2 log T cell reduction, an average 50 kg patient would receive 2×10^5 (1.4 to 2.8×10^5) T cells per kg. Thus, based on Kernan's studies, data presented here suggest that depletion of BM T cells by T101-IT plus amantadine may be effective in preventing severe grade III-IV GVHD.

Amantadine is a licensed drug employed for prophylaxis of influenza A virus infections in humans.¹⁹ The in vitro concentrations found to be active in present experiments cannot be achieved in the blood of patients treated with amantadine.²⁰ For this reason we are now investigating whether amantadine analogues will prove active at clinically occurring concentrations. T101-IT in the presence of non-toxic amantadine concentrations prompts its immediate use for a more efficient ex vivo depletion of human bone marrow T lymphocytes.

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Possible Absence of Common Polymorphisms in Coagulation Factor IX Gene in Japanese Subjects

By Tetsuhito Kojima, Mitsune Tanimoto, Tadashi Kamiya, Yuichi Obata, Toshitada Takahashi, Ryuzo Ohno, Kotoku Kurachi, and Hidehiko Saito

Four distinct intragenic polymorphisms in the coagulation factor IX gene which have been reported to be important for family diagnosis of Caucasian hemophilia B were studied in 51 normal Japanese subjects (21 males and 30 females). High-molecular-weight DNA prepared from peripheral blood lymphocytes were digested with endonuclease, Ddel, MspI, TaqI or XmnI, and were studied by Southern blot analysis with factor IX complementary DNA

as a probe. None of the minor fragments produced by these enzymes was found in the normal Japanese DNA samples tested, although the probe detects minor allelic forms in control Caucasian DNA samples. Our data suggest that the frequent polymorphic sites found in Caucasians are possibly absent in the Japanese population.

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COAGULATION factor IX (Christmas factor) is a vitamin K-dependent plasma protein that participates in the early phase of blood clotting. The absence or functional deficiency of this protein causes a severe bleeding tendency known as hemophilia B or Christmas disease. The recent isolation of a complementary DNA (cDNA) sequence coding for this protein¹⁻³ and the gene for factor IX^{4,5} has opened a new era in the genetic study of hemophilia B. The disease results from heterogeneous genetic causes including single amino acid substitutions,⁶ partial gene deletions,^{7,8} and intronic point mutations.⁹ Besides the structural analysis of abnormal genes, the discovery of restriction fragment length polymorphisms (RFLPs) within the factor IX gene¹⁰⁻¹⁴ has facilitated the clinical application of these polymorphic fragments as genetic markers for family diagnosis such as carrier detection and possible prenatal diagnosis.

Hemophilia B is one of the most common hereditary coagulation disorders in the Japanese population. The occurrence of the disease among Japanese is almost equal to that in whites.¹⁵ We have recently initiated the study of RFLPs in Japanese hemophilia B patients and their families. Interestingly, the majority of normal Japanese subjects was found to possess only the major RFLP fragments of the factor IX gene. Our results suggest that there may be another type of limitation of molecular genetic techniques in family diagnosis of hemophilia B, in addition to the limitations already pointed out by Graham et al.¹⁶

MATERIALS AND METHODS

Twenty milliliters of peripheral blood was drawn from 51 normal Japanese subjects (21 males and 30 females) and 5 normal white subjects (2 males and 3 females) after informed consent. High-molecular-weight DNA was prepared from peripheral blood lymphocytes by sodium dodecyl sulfate (SDS)-proteinase K method followed by phenolchloroform extraction as described elsewhere.¹⁷

Ten to twenty micrograms aliquots of DNA were digested with endonuclease Ddel, MspI, TaqI, or XmnI (Boehringer Mannheim, Indianapolis). DNA fragments were electrophoresed on 0.6% to 1.5% agarose gels and transferred onto nitrocellulose filters (Schleicher & Schuell, Inc, Keene, NH) by Southern's method.¹⁸ Hybridization was carried out with ³²P-labeled factor IX cDNA probe, pHFIX (1.45kb PstI fragment)¹ for 16 hours at 65 °C. The filters were washed and subjected to autoradiography with Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

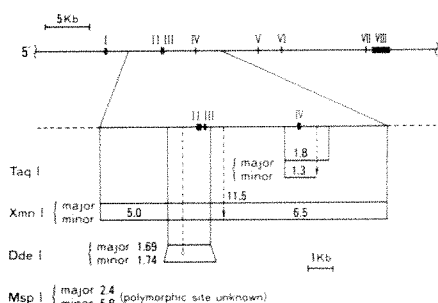


Fig 1. The location of factor IX intragenic common RFLP sites analyzed in this study. TaqI and XmnI sites that produce minor allelic forms are indicated with solid arrows (▼). Insertion polymorphism region detected by Ddel is shown with an open arrow (Δ).

RESULTS

Factor IX gene RFLPs analyzed in this study are shown in Fig 1. Genomic DNA samples from 51 normal Japanese subjects (21 males and 30 females) were digested with TaqI endonuclease and then analyzed with factor IX cDNA probes. While control genomic DNA from two white subjects showed the TaqI minor allelic form (1.3kb fragment), no minor fragment in 20 Japanese genomic DNA samples was detected under the same experimental conditions (Fig 2A).

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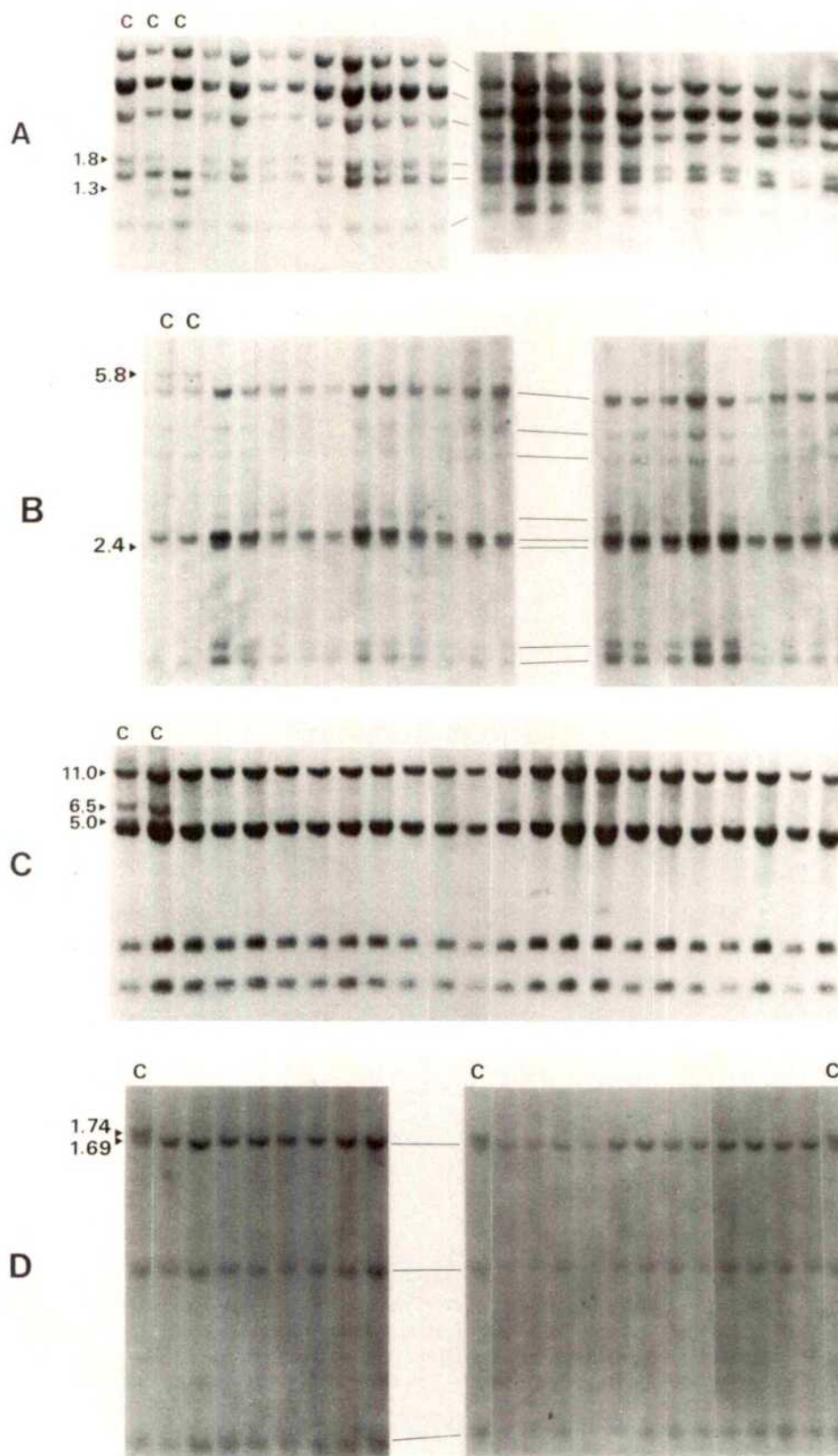


Fig 2. Southern blot analysis of high molecular-weight DNA from normal Japanese and Caucasian subjects with factor IX cDNA probe. Four restriction endonucleases, TaqI (A), MspI (B), XmnI (C), and DdeI (D) were used for digestion of DNA. Factor IX cDNA probe detects 1.3kb TaqI minor fragment in two of three control Caucasian (c) DNA samples (10 to 20 μ g DNA per lane) tested. 5.8kb MspI, 6.5kb/5.0kb XmnI, and 1.74kb DdeI minor forms are observed in Caucasian DNA samples. Minor allelic forms are, however, not observed in any Japanese DNA samples tested. Agarose gel electrophoresis was performed at 1.0% to 0.7% (TaqI), 0.6% (MspI), 0.7% (XmnI), and 1.5% (DdeI).

Similarly, both the 5.8kb MspI and 6.5kb/5.0kb XmnI minor alleles were observed among the white control samples (Figs 2B and C). All the Japanese DNA samples in the same blot, however, possessed neither the MspI nor XmnI minor fragment polymorphism. DdeI digestion was also carried out, but no 1.74 kb minor allele was found among the normal Japanese subjects analyzed (Fig 2D).

The incidence of these four enzyme-derived minor alleles in 81 Japanese X chromosomes was compared with that in white X chromosomes previously described in the literature^{10,12,13} (Table 1). The results indicated that the Japanese factor IX gene is significantly different from the white factor IX gene in terms of the four enzyme polymorphisms analyzed ($P < 0.01$).

DISCUSSION

We demonstrated that the previously described intragenic RFLPs in the factor IX gene produced by endonuclease DdeI, MspI, TaqI, and XmnI are not found in 51 normal Japanese subjects. While DdeI polymorphism is produced by a 50 base pair insert in intron A, the other three RFLPs are generated by point mutations (Fig 1). The experimental conditions employed in this study permitted detection of minor alleles in Caucasian DNA samples digested with each restriction enzyme (Fig 2). To avoid the geographical influences of normal Japanese DNA samples, unrelated random blood donors were chosen for this study, from 10 different prefectures situated in two of four major islands in Japan. The data obtained in this study clearly indicated that common factor IX gene RFLPs among Caucasians are possibly absent in Japanese. Similar results have also been

Table 1. Frequency of Intragenic Polymorphisms in Factor IX Gene in Japanese and Caucasian Subjects

Enzymes	Japanese	Caucasian	P Value
TaqI (1.3kb)	0 n* = 81	0.29 n = 77	$P < 0.01$
MspI (5.8kb)	0 n = 81	0.20 n = 46	$P < 0.01$
XmnI (6.5kb/5.0kb)	0 n = 81	0.29 n = 72	$P < 0.01$
DdeI (1.74kb)	0 n = 81	0.24 n = 74	$P < 0.01$

The incidence of minor alleles in the factor IX gene in Japanese subjects was compared with that in Caucasians. The data for Caucasians were collected from the literature (ref 10 for TaqI, ref 13 for MspI, ref 12 for XmnI, and ref 12 for DdeI). Statistical analysis was done by Fisher's exact test.

*Number of X chromosomes analyzed.

obtained in the Asian population including Japanese, Chinese, and Filipinos in Seattle (Chen S-H, Kurachi K: unpublished observation).

Recent isolation of the factor IX cDNA sequence and successful cloning of the factor IX gene has brought a new era in the clinical diagnosis of hemophilia B. After discovery of the informative TaqI RFLP, which is located in intron D,¹¹ several other RFLPs have been found. These polymorphisms have already been used for clinical diagnosis for Caucasian hemophilia B families.¹²⁻¹⁴ There are, however, several limitations in molecular genetic diagnosis for hemophiliacs as pointed out recently.¹⁶ The results described here suggested that there is one more type of limitation for diagnosis of hemophilia B in certain races, such as the Japanese.

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CONCISE REPORT

Effect of Human Recombinant Granulocyte Colony-Stimulating Factor on Hematopoietic Injury in Mice Induced by 5-Fluorouracil

By Michiya Shimamura, Yukio Kobayashi, Akira Yuo, Akio Urabe, Tetsuro Okabe, Yoshinori Komatsu, Seiga Itoh, and Fumimaro Takaku

Intravenous (IV) administration of 5-fluorouracil (5-FU) caused severe hematopoietic injury in mice. Daily administration of purified human recombinant granulocyte colony-stimulating factor (rG-CSF) accelerated recovery from neutropenia in these injured mice. Granulocyte-macrophage progenitors (CFU-GM) in spleen were markedly increased

by rG-CSF, while rG-CSF was less effective on the regeneration of CFU-GM in femoral marrow. The accelerated recovery from neutropenia in the mice might be mainly due to the stimulation of granulopoiesis in spleen by rG-CSF.

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IT IS WELL KNOWN that the proliferation and differentiation of hematopoietic progenitors are directly regulated by the stimulating factors specific to each lineage of the progenitors,¹ respectively. Recently the genes encoding some human hematopoietic factors, such as erythropoietin,² granulocyte colony-stimulating factor (G-CSF),³ and granulocyte-macrophage colony-stimulating factor (GM-CSF),⁴ have been cloned. Our laboratories have also succeeded in producing human recombinant G-CSF (rG-CSF) independently.⁵ In vivo activities of the preparations of rG-CSF have been already demonstrated.^{5,6} In consideration of clinical applications of rG-CSF, we have been trying to estimate the therapeutic effect of rG-CSF on an animal model of neutropenia induced by anti-cancer drugs. In this paper we report an enhancing effect of rG-CSF on the granulopoietic recovery in mice injured hematopoietically by 5-fluorouracil (5-FU).

MATERIALS AND METHODS

Human G-CSF was purified to homogeneity from a protein-free culture cell line, Balanche-1,⁷ and the amino-terminal sequence of G-CSF was determined by a gas-phase sequencer (unpublished results). Using poly (a)⁺RNA prepared from human macrophages, a cDNA library was constructed by the methods of Okayama and Berg.⁸ The resulting cDNA library was screened by the synthetic oligonucleotide probes, which were predicted by the amino-terminal sequence. The amino acid sequence deduced from the cloned cDNA was identical to that reported by Souza et al.³ The cDNA was expressed in *Escherichia coli* K12 MM294 by construction of an expression plasmid for G-CSF using pGEL-1 containing the *trp* promoter,⁹ and the mature protein was purified to homogeneity as previously reported.¹⁰ Details on cloning and expression of the cloned cDNA will be described elsewhere (unpublished results). Units of rG-CSF activity were defined according to the method of Nicola et al.,¹¹ ie, the rG-CSF concentration required for half-maximal stimulation of colony formation by 75,000 BDF₁ mouse (Charles River, Japan) bone marrow cells in a semisolid culture medium was defined as 50 U of activity. As a measurement of purity, the purified rG-CSF was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis according to the method of Laemmli¹² and was tested for endotoxin contaminations using a limulus amoebocyte lysate assay.³ The purified rG-CSF was dissolved in endotoxin-free normal saline for subcutaneous (SC) injection to mice in doses of 5×10^4 to 2×10^5 U/mouse/day.

Female BDF₁ mice (7 weeks old) were injected intravenously (IV) with 5-FU (200 mg/kg body weight; Sigma Chemical, St Louis) on day 0. Daily SC administration of rG-CSF was carried out from day 0. The 5-FU-treated control mice received only saline. In one experiment mice were sampled randomly from each group on days 5

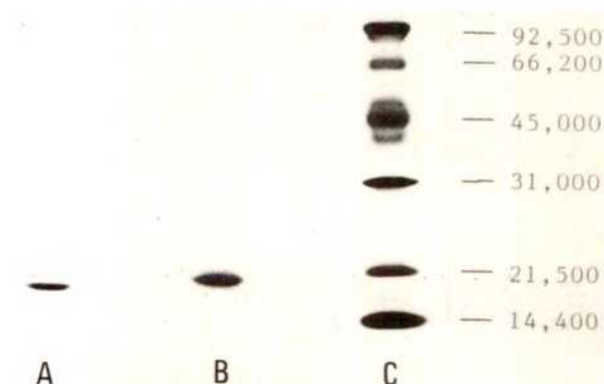


Fig 1. Analysis of human G-CSF and rG-CSF on an SDS-15% polyacrylamide gel stained with silver. (A) 2 µg of rG-CSF; (B) 2 µg of G-CSF from Balanche-1 cells; (C) molecular weight markers.

7, 9, 11, and 13, and blood was collected from the orbital sinus for neutrophil counts. Nucleated cells in blood were counted using a Coulter counter (model ZF; Coulter Electronics, Hialeah, FL). Blood smears were stained with Wright-Giemsa for differential counts of leukocytes. A femur and spleen were collected from each mouse under sterile conditions. Using 23-gauge needles, femoral marrow cells or spleen cells were harvested in 2 or 5 mL of α -medium (Flow Laboratories, McLean, VA). The cells were counted using a Coulter counter and were washed by centrifugation at $400 \times g$. Granulocyte-macrophage progenitors (CFU-GM) in femoral marrow cells or in spleen cells were assayed in the semisolid agar culture system¹⁴ as follows: The cells (5×10^4 /plate) were plated in 0.5-mL aliquots of α -medium containing 20% fetal bovine serum (Flow Laboratories, North Ryde, NSW, Australia), 0.3% agar, and 10% WEHI-3 conditioned medium as a source of colony-stimulating factor.¹⁵ These cultures were carried out in 24-multiwell tissue-

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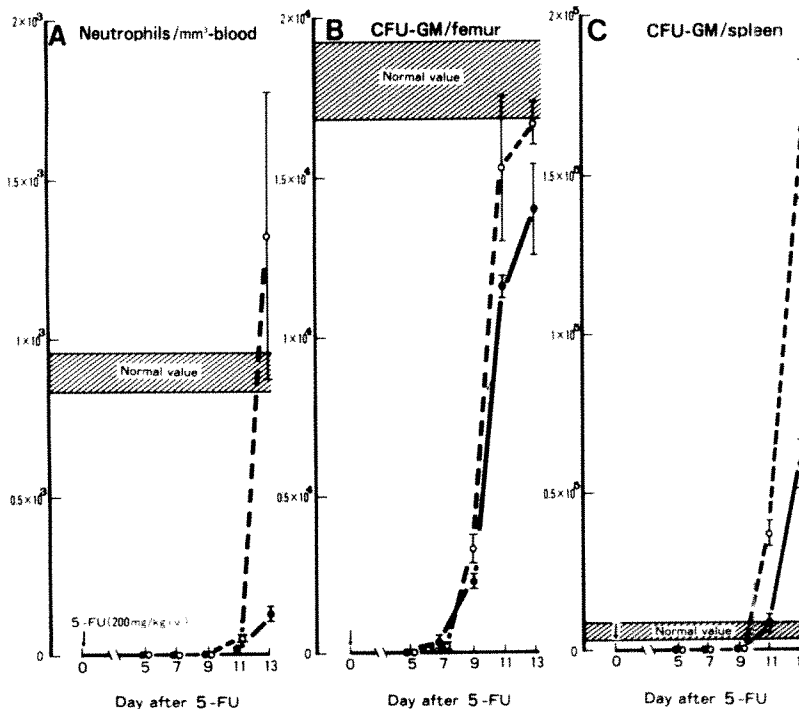


Fig 2. Quantitative changes of neutrophils in blood. (A) CFU-GM in femoral bone marrow; (B) CFU-GM in spleen; and (C) CFU-GM in the mice after receiving 5-FU on day 0. A group of mice (○) were daily administered with rG-CSF (1×10^5 U/mouse/d) from day 0. Another group of mice (●) received only saline. Data represent mean value $\pm$ SE obtained from four mice in each data point.

culture plates (Corning 25820; Iwaki Glass, Japan). Duplicate cultures were made from each murine femur or spleen and were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. After seven days of incubation, clonal colonies consisting of more than 50 cells were scored using an inverted microscope. In another experiment, blood (less than 30 μ L) was collected successively on days 9, 11, and 13 from the tail vein of each mouse for neutrophil counts.

RESULTS

The purified rG-CSF was found to be homogenous by SDS-polyacrylamide gel electrophoresis (Fig 1) and to have an apparent molecular weight of 17,400, whereas human G-CSF purified from cell culture⁷ migrated with an apparent molecular weight of 18,000. The purified rG-CSF had a specific activity of 1×10^8 U/mg of protein and contained less than 2.0 ng of endotoxin/mg of protein. This means that the *in vivo* studies were carried out in an almost endotoxin-free system.

Quantitative changes of neutrophils in blood and those of CFU-GM in femur or spleen of the mice injected with 5-FU are summarized in Fig 2. 5-FU caused severe neutropenia and a significant decrease of CFU-GM. More than 13 days were required for the full recovery from neutropenia in the 5-FU-treated mice. On the contrary, the daily administration of rG-CSF (1×10^5 U/d) to the 5-FU-treated mice caused a marked elevation in neutrophil counts on days 11 and 13 (313% and 1,019% of each paired control, respectively) as shown in Fig 2A. In comparison with the mild enhancing effect of rG-CSF on CFU-GM in femur (Fig 2B), rG-CSF administration showed a marked increase in CFU-GM in spleens on days 11 and 13 (498% and 278% of each paired control, respectively) as shown in Fig 2C. The recovery

from neutropenia in 5-FU-treated mice was accelerated by 5×10^4 to 2×10^5 units of rG-CSF in a dose-dependent manner (Fig 3).

DISCUSSION

Several laboratories have succeeded in large-scale production of hematopoietic factors from their cloned genes. Some of these recombinant factors are expected to be used as stimulators of hematopoietic restoration from anti-cancer drugs or irradiation-induced hematopoietic injury.¹⁶ Our observations described here support the possibility of therapeutic applications of rG-CSF in neutropenia caused by

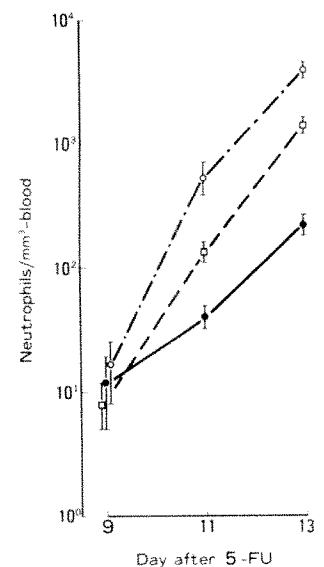


Fig 3. Quantitative changes of neutrophils in blood of the mice after receiving 5-FU on day 0. Mice in each group (three mice/group) daily received 2×10^5 U/d of rG-CSF (○), 5×10^4 U/d of rG-CSF (□), or only saline (●). Blood less than 30 μ L was successively collected from tail vein of each mouse for neutrophil counts. Data represent mean value $\pm$ SE.

cancer chemotherapy. 5-FU in a dose of 200 mg/kg body weight caused severe neutropenia in mice. As shown in the results, it seemed that there were two stages in the process of granulopoietic recovery after being injured by 5-FU: The stage of regeneration of CFU-GM in bone marrow preceded

that of a CFU-GM increase in spleen. The administration of rG-CSF was much more effective in this second stage than in the first one. Thus, rG-CSF stimulated in vivo granulopoiesis to accelerate recovery from neutropenia in an endotoxin-free system.

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Terminal Deoxynucleotidyl Transferase Expression Can Precede T Cell Receptor β Chain and γ Chain Rearrangement in T Cell Acute Lymphoblastic Leukemia

By James M. Greenberg and John H. Kersey

The nuclear enzyme terminal deoxynucleotidyl transferase (TdT) is thought to contribute to the diversity of certain immunoglobulin and T cell receptor gene rearrangements through the addition of random nucleotides at their variable (V)-joining (J) region junctions. An acute lymphoblastic leukemia (ALL) with an immature T cell phenotype (CD7⁺, CD5⁺, CD1⁺, CD2⁺, CD3⁺, CD4⁺, CD8⁺) was found to be TdT⁺ with germline immunoglobulin heavy chain, T

cell receptor β chain, and T cell γ chain genes. The data indicate that TdT expression can precede T γ and T β rearrangement during T lymphoid ontogeny consistent with its proposed association with the T cell receptor rearrangement process. Southern analysis of certain cases of T-ALL may not result in the detection of a monoclonal population of cells.

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LYMPHOID progenitor cells in the bone marrow are known to express the nuclear enzyme terminal deoxynucleotidyl transferase (TdT).¹ This enzyme catalyzes the random addition of free deoxynucleotides onto the 3'-hydroxyl terminus of a nucleotide without the presence of a primer.² Experimental evidence suggests that TdT may be responsible for the insertion of extra random nucleotides between variable (V), diversity (D), or joining (J) regions during the rearrangement of immunoglobulin heavy chain or T cell receptor β chain genes during lymphoid ontogeny. These added sequences have been designated as N regions and are thought to contribute to the potential diversity of the antigen receptors encoded by these genes.^{3,4}

Three structurally related rearranging genes are associated with T cells. Two of these genes code for the polypeptide chains that compose the T cell receptor α/β heterodimer.⁴⁻⁷ The third, T γ , is rearranged and expressed prior to T β rearrangement during lymphoid ontogeny.⁸⁻¹⁰ Recent evidence suggests that T γ encodes a novel CD3-associated antigen receptor polypeptide on a subpopulation of human lymphocytes.^{11,12} T γ has also been shown to contain N regions that contribute to its diversity.¹³

A recent study indicates that TdT expression occurs during early T cell development.¹⁴ However, the precise temporal relationship between TdT gene expression and T cell receptor gene rearrangement during ontogeny is currently unknown. This sequence is of some importance in light of the emerging data on the early development of T γ rearrangement and expression reported by ourselves and others.⁸⁻¹² In this report, we describe an acute lymphoblastic leukemia of prothymocyte phenotype that has TdT expres-

sion but germline T γ , T β , and immunoglobulin genes. Our data suggest that TdT gene expression begins prior to T β and T γ rearrangement in T lymphoid progenitor cells. The results also indicate that early T cell leukemias may have germline T β and T γ genes.

MATERIALS AND METHODS

Case history. The leukemic cells described in this study were obtained from the peripheral blood and bone marrow of a 26-year-old female who presented with leukocytosis, thrombocytopenia, and anemia during preoperative evaluation for an elective cesarean section. Bone marrow and peripheral blood contained 95% and 80% lymphoblasts, respectively, at the time of diagnosis. There was no evidence of a thymic or mediastinal mass. The leukemic cells were myeloperoxidase, chloroacetate esterase, and Sudan black B negative. Cytochemical staining with nonspecific esterase and acid phosphatase was focal paranuclear positive suggestive of T lymphoblasts.¹⁵

A diagnosis of T cell acute lymphoblastic leukemia, FAB L2, was made on the basis of the morphologic, cytochemical, and immunophenotypic analysis of the leukemic lymphoblasts (Table 1). The patient received induction chemotherapy with vincristine, daunorubicin, prednisone, L-asparaginase, and methotrexate and has been maintained in remission with additional chemotherapy for 25 months.

Peripheral blood and bone marrow specimens were obtained following informed consent and utilized under guidelines established by the University of Minnesota Committee on the Use of Human Subjects in Research.

Cells. Suspensions of mononuclear cells containing leukemic lymphoblasts were prepared from peripheral blood or bone marrow by centrifugation through a discontinuous Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient. The mononuclear cells at the Ficoll-Hypaque interface were resuspended in RPMI medium supplemented with 5% fetal calf serum. Cell suspensions were either used immediately or frozen in liquid nitrogen following the addition of 10% dimethyl sulfoxide to the medium.

Immunophenotypic analysis. Cells were analyzed for the presence of various cell surface antigens associated with hematopoietic cells (Table 1).¹⁶⁻³⁴ Following two washes in Ca⁺⁺, Mg⁺⁺-free phosphate-buffered saline, 10⁶ cells were mixed with a saturating concentration of murine or rabbit monoclonal antibody and allowed to incubate for 30 minutes at 4 °C. The cells were then washed twice and incubated for an additional 30 minutes at 4 °C with either a goat fluorescein-labeled antibody (FITC-G α M) that detected an initial murine monoclonal antibody or a goat rhodamine-labeled antibody (RITC-G α R) that detected an initial rabbit antibody. After three washes, the cells were analyzed with an automated fluorescence-activated flow cytometer (Ortho Spectrum III cytofluorograf, Ortho

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Table 1. Immunophenotypic Characteristics of Leukemic Cells

Antibody	Cluster Designation	Positive (%)	Reference
anti-TdT	—	89	16
3A1	CD7	97, 95	17
TA-1	—	86, 73	18
T101	CD5	67, 66	19
OKT6	CD1	32, 26	20
35.1	CD2	34, 22	21
OKT3	CD3	7, 2	20
Leu-3	CD4	7, 5	22
OKT8	CD8	4, 2	20
HLA-DR	—	18, 8	23
BA-1	CD24	1, 0	24
BA-2	CD9	5, 1	25
BA-3	CD10	4, 1	26
B1	CD20	13	27
B4	CD19	19	28
MCS-2	CDw13	5	29
MY3	—	0	30
MY4	CDw14	2	31
MY8	—	1	31
MY9	—	1	32
3C5	—	2	33
anti-TAC	CD25	0	34

Diagnostic Systems, Raritan, NJ, or FACS IV, Beckton-Dickinson, Mountain View, CA). Appropriate controls using cells stained only with FITC- α M were used to assess autofluorescence, and control ascites fluid was used to determine the extent of background staining. The flow cytometer generated a fluorescence profile for each antibody tested allowing the percentage of antigen-positive cells to be determined.

DNA extraction. High-molecular-weight DNA is extracted from bone marrow or peripheral blood mononuclear cells using a nuclei extraction technique as described by Bakhshi et al.³⁵ Isolated nuclei are lysed with sodium dodecyl sulfate (SDS) in the presence of proteinase K. The resultant viscous DNA solution is then extracted several times with phenol/chloroform/isoamyl alcohol (25:24:1) until the aqueous-organic interface is clear. Following a final extraction with chloroform/isoamyl alcohol (24:1), the DNA is ethanol-precipitated with 5 mol/L NaCl and resuspended in a small amount of TE buffer (10 mmol/L Tris Cl, 1 mmol/L EDTA, pH 7.4).

The concentration of DNA samples is determined by measurement of the OD₂₆₀. DNA samples extracted by the aforementioned methods routinely give OD₂₆₀:OD₂₈₀ ratios of 1.7 to 2.0.

Probes. Rearrangements of T γ are detected with an 800 bp genomic DNA probe containing sequences that hybridize to both joining regions of T γ . This probe was originally obtained from the laboratory of Dr J. Seidman, Harvard Medical School. It detects two germline EcoRI restriction fragments (3.3 kb and 1.8 kb).³⁶ Rearrangements of the β chain of the T cell receptor are detected with a 770 bp cDNA probe that recognizes the constant region of T β obtained from Dr T.W. Mak, Ontario Cancer Institute. The T β probe hybridizes to two germline EcoRI restriction fragments (11 kb and 4.0 kb) or one germline BamHI restriction fragment (24 kb).³⁷ Immunoglobulin heavy chain gene rearrangements were detected with a probe for the constant regions of the gene (C μ) obtained from Dr S.J. Korsmeyer, National Institutes of Health, Bethesda, MD. It detects a single 17 kb BamHI restriction fragment in germline DNA.³⁸

Southern blot analysis. Gene rearrangements are detected using the gel transfer technique first described by Southern.³⁹

High-molecular-weight genomic DNA is digested to completion using a selected restriction endonuclease. The restriction fragments are then size fractionated by electrophoresis through 0.8% agarose and transferred from the gel to a nylon filter (Zetabind, AMF Cuno) in 20 $\times$ SSC (1 $\times$ SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.4). After washing and prehybridization, the filters are hybridized in 50% formamide at 42 °C for 20 to 36 hours using probes labeled to high specific activity with ³²P by the random primer method.⁴⁰ The filters were then washed for 15 minutes in 2 $\times$ SSC, 0.1% SDS followed by one to two hours in 0.1 $\times$ SSC, and 0.1% SDS at the appropriate stringency for the probe being used. Filters were exposed to film (XAR-5, Kodak), with one intensifying screen for two to seven days at -70 °C.

RESULTS

Mononuclear cells from bone marrow and peripheral blood were subjected to immunophenotypic analysis. The results obtained with each monoclonal antibody are summarized in Table 1. The malignant lymphoblasts had an immunophenotypic profile corresponding to immature thymocytes or prothymocytes. The cells were strongly positive for TdT, the CD7 antigen (3A1), the CD5 antigen (T101), and the antigen detected by TA-1. Significant but lower levels of expression of the CD1 (OKT6) and CD2 (35.1) antigens were also detected. There was no significant staining with monoclonal antibodies directed against cell surface antigens associated with B lymphoid or myeloid lineage cells. Monoclonal antibody 3C5, which detects a cell surface antigen associated with very immature hematopoietic progenitor cells,³³ did not bind to the malignant cells.

Southern blot analysis was performed in order to detect rearrangements of IgH, T α , and T β in the malignant cells (Fig 1). With each probe, a germline pattern was detected when directly compared with germline DNA obtained from placenta. We verified the T β germline pattern seen in Fig 1 following EcoRI digestion by performing the same analysis with BamHI and HindIII (data not shown).

We, and others, have noted occasional instances when the T γ J probe detects rearrangement following BamHI digestion but not EcoRI.^{10,41} Therefore, patient DNA was also analyzed for T γ rearrangement following BamHI digestion and found to be germline.

DISCUSSION

Our results demonstrate that TdT expression can precede T cell receptor gene rearrangement in lymphoid ontogeny. This is, to our knowledge, the first report of a TdT-positive T lymphoid malignancy with germline T β and T γ and immunoglobulin genes. The notion that TdT expression may precede VJ (or VDJ) recombination is compatible with its proposed role in the generation of N region diversity during the gene rearrangement process.

The composite phenotype in this case is most consistent with that of an early thymocyte or prothymocyte. The lack of reactivity with a battery of myeloid and B lymphocyte lineage antigens, the lack of staining with myeloid cytochemical stains combined with expression of TdT, CD7, CD5, CD1, and CD2, all strongly argue for an early T lymphocyte lineage.

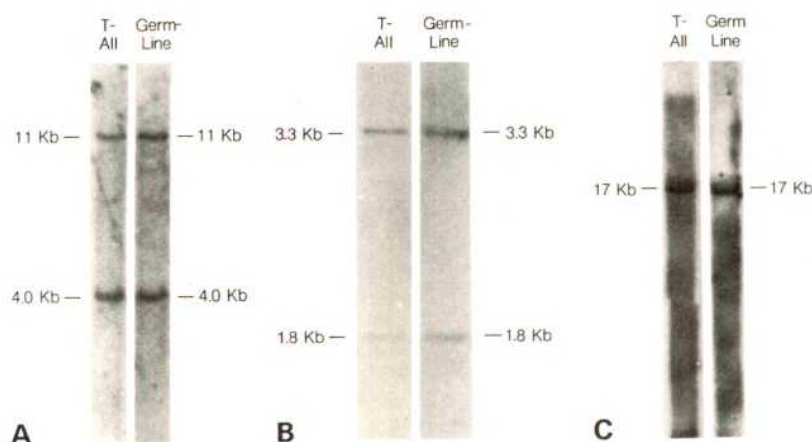


Fig 1. Southern blot analysis of T-ALL DNA. DNA was digested with *Eco*R1 and probed with T β (A); *Eco*R1 probed with T γ (B); and *Bam*H1 probed with C μ (C). Each T-ALL lane is accompanied by an identical Southern analysis of germline DNA obtained from human placenta. The sizes of the restriction fragments detected by each probe are indicated adjacent to each lane.

A recent report indicates that TdT expression as detected by an immunofluorescence assay may be spurious in PHA-stimulated peripheral blood mononuclear cells.⁴² Because cells were not available for a biochemical determination of TdT activity, we cannot rule out the possibility that our immunofluorescence assay may be detecting a cross-reacting protein lacking TdT activity. However, the morphologic and immunophenotypic characteristics of leukemias of an immature lymphoid phenotype have shown good concordance between immunofluorescence and biochemical determinations of TdT expression.⁴³

Recent reports suggest that CD7 (3A1) expression precedes T β rearrangement in T-ALL.^{14,44} Our findings indicate that certain T cell-associated cell surface antigens such as CD1, CD2, and CD5 as well as CD7 can be expressed prior to T γ as well as T β rearrangement. Although the function of these antigens is not known, their expression in a lymphoid cell with an immature (prothymocyte-type) T phenotype prior to gene rearrangement suggests that some or all may be involved in the thymic homing process.

The majority of lymphoid malignancies have a phenotypic counterpart in normal bone marrow, thymus, or peripheral

blood.⁴⁵ Lymphoid cells with immunophenotypic characteristics similar to those in our patient (CD7⁺, TdT⁺) have been detected in the peripheral blood and bone marrow of normal individuals.⁴⁶ Thus, the leukemic cells are likely an accurate representation of normal lymphopoiesis. However, occasional leukemias may not faithfully reflect early hematopoietic events.⁴⁷ The exceedingly low frequency of normal cells with this phenotype⁴⁶ makes DNA analysis of these cells not practical.

Rearrangements of T cell receptor genes (T β and T γ) can be detected by Southern blot analysis and have been used to detect clonal populations of malignant T lymphoid cells from bone marrow, peripheral blood, or lymph nodes.⁴⁸⁻⁵⁴ The results described above indicate that certain cases of T-ALL may not have detectable T cell receptor gene rearrangements even though they display a relatively unambiguous T phenotype. In such cases, Southern blot analysis to detect clonal populations of malignant cells will not provide useful information. Thus, adequate characterization of lymphohematopoietic malignancies continues to require a multidisciplinary approach including morphologic, immunophenotypic, and cytochemical analysis.

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Marrow Purging in Autologous Bone Marrow Transplantation for T-Lineage Acute Lymphoblastic Leukemia: Efficacy of Ex Vivo Treatment With Immunotoxins and 4-Hydroperoxycyclophosphamide Against Fresh Leukemic Marrow Progenitor Cells

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A lymphoblast progenitor cell assay was used to evaluate the antileukemic efficacy of marrow-purging protocols that employed intact ricin immunotoxins (IT) and 4-hydroperoxycyclophosphamide (4-HC) against clonogenic primary T-lineage marrow blasts freshly obtained from 12 T-lineage acute lymphoblastic leukemia (ALL) patients. Residual T-lineage blast colonies were observed after treatment with 1 µg/mL T101 (anti-CD5)-Ricin (R) + G3.7 (anti-CD7)-R in eight of 12 cases and after 100 µg/mL 4-HC

in six of nine cases. By comparison, a combination of IT and 4-HC proved very effective against T-lineage leukemic progenitor cells, and no residual blast colonies were observed in any of the eight cases studied. We conclude that future trials should consider combined treatment protocols such as IT + 4-HC for more effective purging of autologous marrow grafts.

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THE PROGNOSIS of patients with T-lineage acute lymphoblastic leukemia (ALL) who experience bone marrow relapse while on maintenance therapy is poor. High-dose chemotherapy in combination with total body irradiation, followed by rescue with allogeneic or autologous bone marrow, is currently being investigated in a number of centers as an alternative to conventional chemotherapy for these high risk patients.¹⁻⁴

Autologous bone marrow transplantation (BMT) circumvents graft-v-host disease and results in a lower peritransplant morbidity or mortality than allogeneic BMT.¹ Autologous BMT does not require a histocompatible sibling donor and therefore a larger number of patients can meet the eligibility criteria for autologous than for allogeneic BMT. However, the likely presence of contaminating leukemic progenitor cells in the autologous marrow grafts, especially when the marrow is harvested during second or subsequent remission, or first remission prior to leukemic relapse, is a major concern.

A variety of immunologic^{5-10,13} and pharmacologic¹¹⁻¹³ strategies are being evaluated for effective purging of residual leukemic cells from autografts. Although most purging protocols have proved effective against established leukemic cell lines,⁵⁻¹¹ none have been tested against clonogenic marrow blasts taken directly from ALL patients. Therefore, there is no evidence that they can effectively eliminate residual ALL blasts from autografts. In the absence of data on fresh leukemic blast progenitors from ALL patients, it is difficult to evaluate and improve the efficacy of ex vivo marrow purging strategies.

Herein, we report the results of a preclinical study examining the efficacy of three distinct purging protocols at the level of T-lineage leukemic progenitor cells, using fresh leukemic marrow samples from 12 T-lineage ALL patients and blast progenitor cell assays. Specifically, we compared the antileukemic efficacy of (1) a cocktail of T101-R (anti-CD5) and G3.7-R (anti-CD7) IT, (2) 4-HC, as well as (3) a combination of both protocols, ie, combined immunochemotherapy against fresh clonogenic T-lineage ALL marrow blasts. We show that purging was not complete after IT or 4-HC treatment, and combined immunochemotherapy proved more effective than IT alone or 4-HC alone in many cases.

MATERIALS AND METHODS

Patient material. Twelve patients with T-lineage ALL were included in this study. Clinical and diagnostic data are summarized in Table 1. Bone marrow aspirate samples were obtained by conventional methods. Morphological classification was performed on Wright-Giemsa stained slides using a modification of the original French-American-British (FAB) classification. Blast cells were isolated by a single centrifugation on Ficoll-Hypaque (1.077 g/cm³). Marker analyses by indirect immunofluorescence (IF) and flow cytometry were performed using a panel of monoclonal antibodies (mAb) that define T-lineage (ie, 35.1, T101, G3.7) and B-lineage (ie, BA-1, BA-3) leukocyte differentiation antigens as previously described (Table 1).⁹

Colony assay for T-lineage ALL progenitor cells. Freshly obtained bone marrow blasts were cultured using a modification of a recently described assay system that allows reproducible proliferation and colony formation of fresh marrow blasts from patients with

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Table 1. Clinical and Diagnostic Data on Patients with T-Lineage ALL

UPN	Sex/Age (yr)	Diagnosis (FAB)	New Case or Relapse	Bone Marrow % Blasts	Immunophenotype of Marrow Blasts % Positive*				
					CD2 35.1	CD5 T101	CD7 G3.7	CD10 BA-3	CD24 BA-1
1	M/16	L1	New	80	97	82	93	6	1
2	M/5	L2	Relapse	81	73	83	91	13	5
3	M/2	L1	New	76	85	84	26	0	0
4	M/9	L1	New	94	54	49	89	0	0
5	M/8	L1	New	100	96	88	92	0	2
6	F/12	L1	New	76	92	81	90	12	2
7	M/10	L1	New	92	78	67	94	2	10
8	M/7	L1	New	80	74	50	80	0	1
9	M/3	L1	New	90	61	81	88	0	6
10	M/11	L1	New	98	96	87	93	2	0
11	M/15	L2	New	75	86	34	51	0	2
12	M/15	L1	Relapse	100	95	85	83	43	1

*The immunological marker profiles of marrow blasts were determined by indirect IF and flow cytometry as described.⁹

Results are expressed as the percentage (%) of blasts positive for each marker.

B-cell precursor ALL.⁹ In brief, density gradient-isolated and adherent cell-depleted fresh marrow blasts from T-lineage ALL patients were suspended after treatment with various purging reagents in alpha-MEM (minimum essential medium) supplemented with 0.9% methylcellulose, 15% fetal calf serum (FCS), 15% platelet-rich fresh human plasma, 10% phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM), 1,000 U/mL recombinant interleukin 2 (rIL-2) (generously provided by Dr Michael Widmer and the Immunex Corp, Seattle), and 50 μ mol/L 2-mercaptoethanol (2-ME). Duplicate 1-mL samples containing 10^5 blasts were cultured in 35-mm Petri dishes for seven days at 37 °C in a humidified 5% CO₂ atmosphere. On day 7, blast colonies containing >20 cells were counted using an inverted phase microscope with high optical resolution. Subsequently, all colonies were pooled for morphologic, cytochemical, and immunologic analyses of colony blasts. In this assay system, blast colony formation is linear with respect to numbers of cultured cells between 5×10^2 and 5×10^5 leukemic blasts per mL per Petri dish (data not shown). Thus far, colony assays have been performed on 30 T-lineage ALL patients, and blast colony formation was obtained in 20 cases with a median plating efficiency (PE) of 0.372% (Uckun et al, unpublished data). In the 12 cases included in this study, sufficient marrow blasts were obtained to evaluate the antileukemic efficacy of IT and 4-HC. The extent of cytotoxicity was expressed as Log Kill of T-lineage blast progenitor cells and was calculated as Log (PE of sham-treated blasts)/(PE of treated blasts).

Cytochemical and immunologic analysis of colony blasts. Cells from pooled colonies were deposited on slides by cytocentrifugation. The morphology of colony blasts was studied in Wright-Giemsa stained cytospin preparations. Blasts were evaluated for acid phosphatase (AP) activity using the conventional naphthol ASBI phosphoric acid-fast garnet GMC method. For surface marker analyses by indirect immunofluorescence, we used the mAb 35.1, anti-CD2 (T, p50); T101, anti-CD5 (T, p67); and G3.7, anti-CD7 (T, p41) as previously described.⁹ The terminal deoxynucleotidyl transferase (TdT) activity of colony blasts was determined by indirect IF on methanol-fixed cytospin preparations, using immunoadsorbent purified rabbit anti-TdT serum (20 μ g/mL) and FITC-conjugated goat F(ab')₂ antirabbit IgG (60 μ g/mL). These antisera were a generous gift of Dr Frederick Bollum, Uniformed Services University of Health Sciences, Bethesda, Maryland. Furthermore, a standard dual IF staining technique was employed to study the co-expression of nuclear TdT and surface T-lineage antigens. Briefly, methanol

fixed cytospin preparations were first subjected to indirect IF staining with rabbit anti-TdT serum and fluorescein-isothiocyanate (FITC)-conjugated goat F(ab')₂ antirabbit IgG. Subsequently, an equimolar cocktail of T101 and G3.7 mAb at 20 μ g/mL, followed by two washes in phosphate-buffered saline (PBS) and incubation with rhodamine-conjugated goat F(ab')₂ antimouse IgG + IgM (Tago, Burlingame, CA), was used to stain surface CD5 and CD7 determinants.

Source of purging reagents and ex vivo treatment conditions. IT against CD5 and CD7 determinants were synthesized at the University of Minnesota by conjugating T101 (IgG2a, anti-CD5) and G3.7 (IgG1, anti-CD7) mAb to intact ricin as described earlier.^{7,10} GK1.5, which recognizes the p52 surface determinant on murine T cells, was also linked to ricin as previously described⁷ and used as an irrelevant control IT. Details of the chemical conjugation procedure, immunoreactivity, cytotoxicity, and kinetics of action of the generated IT against leukemic T cell lines in protein synthesis inhibition and clonogenic assays, their potentiation by in vitro active cyclophosphamide congeners, as well as their effects on normal committed monopotent and multilineage bone marrow progenitor cells were published in previous reports.^{7,8,10} These IT were extremely toxic against leukemic T cell lines but only minimally toxic to normal bone marrow progenitor cells.^{7,10} Standard ex vivo treatment conditions with IT, 4-HC, and a combination of IT and 4-HC were established in earlier studies.^{7,11-13} They represent the clinical marrow purging protocols in autologous BMT for T-lineage ALL at Minnesota and Baltimore. In brief, cells (10^7 /mL) were treated with (1) 1 μ g/mL IT (an equimolar cocktail of T101-R and G3.7-R) for two hours at 37 °C in the presence of 200 mmol/L lactose (Minnesota protocol), (2) 100 μ g/mL 4-HC for 30 minutes at 37 °C (Baltimore protocol), or (3) a combination of 1 μ g/mL IT (two hours, 37 °C) and 100 μ g/mL 4-HC (30 minutes, 37 °C; 4-HC was added during the last 30 minutes of the IT treatment). Controls included leukemic marrow samples treated with (1) lactose only, (2) an equimolar cocktail of unconjugated T101, G3.7 mAb (0.750 μ g/mL), and free ricin (0.300 μ g/mL), (3) control IT GK1.5-R (1 μ g/mL), or (4) GK1.5-R (1 μ g/mL) + 4-HC (100 μ g/mL).

RESULTS AND DISCUSSION

Clinical and diagnostic features. Twelve patients with T-lineage ALL were studied. Patient data are summarized in Table 1. The patients ranged in age from 2 to 16 years

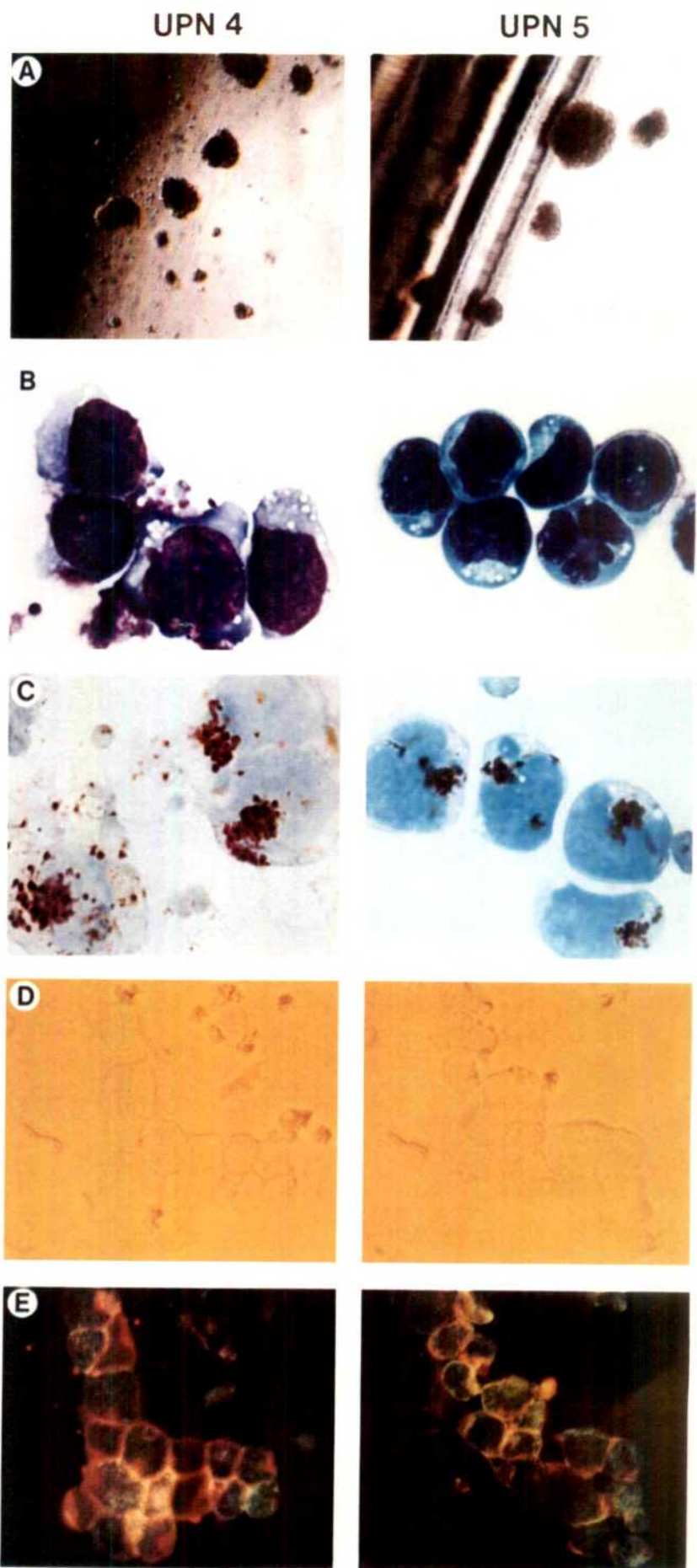


Fig 1. (A) Picture of part of Petri dishes containing compact clusters and colonies of T-lineage lymphoblasts cultured for seven days (x5). (B) Wright-Giemsa stained cells pooled from day 7 blast colonies in (A). Note the blast morphology with a basophilic cytoplasm, irregular nuclear membrane, prominent nucleoli, and vacuolation. (C) Colony blasts were evaluated for acid phosphatase activity. Note the focal paranuclear distribution of the stain. (D) Phase contrast. (E) Dual IF evaluation of colony blasts in the field shown in (D) for nuclear TdT and surface CD5 and CD7.

(median: 9 years). There were 11 males and one female. Two patients were in relapse and ten were newly diagnosed. Ten patients were morphologically classified as L1 ALL, while the subtype of the remaining two was L2. Bone marrow aspiration samples evaluated in the present study contained 75% to 100% blasts (median: 86.5%). No patient expressed the B-lineage-associated surface determinant CD24 recognized by BA-1 mAb. In contrast, the vast majority of cells from all patients reacted with 35.1, T101, and G3.7 mAb, which recognize distinct T-lineage differentiation antigens (Table 1). Only one of the 12 patients (unique patient number [UPN] 12) had >20% CD10/CALLA⁺ marrow blasts. These immunologic marker profiles are consistent with T-lineage affiliation.

Colony assays and analysis of colony blasts. Successful cultures were obtained from fresh marrow samples in all 12 cases. Day 7 blast colonies were compact and contained 50 to 500 cells (Fig 1, Table 2). The PE of blast progenitor cells varied from 0.049% (ie, 49 colonies per 10⁵ BM blasts) to 1.546% (ie, 1,546 colonies per 10⁵ BM blasts) with a median PE of 0.320% (Table 2). The cells from pooled colonies showed >95% viability, as estimated by trypan blue exclusion. They had blast morphology with a basophilic cytoplasm, irregular nuclear membrane, and prominent nucleoli (Fig 1). As shown in Fig 1, colony blasts displayed a strong AP activity in a predominantly focal paranuclear distribution. A minor component of the AP activity was in the form of coarse granules irregularly scattered in the cytoplasm. Blasts from pooled colonies showed a marked heterogeneity in expression of T-lineage-associated surface determinants (Table 2). Despite this heterogeneity, colony blasts in each case were found strongly positive for at least two T-lineage markers. Besides surface marker analyses, colony blasts were also tested for nuclear TdT activity and found strongly positive in all patients. In dual IF analyses, we found that 75% to 100% (median: 85%) of colony blasts in all studied cases were nuclear TdT-positive and strongly reacted with an equimolar cocktail of T101 and G3.7 mAb (Table 2, Fig

1). These marker profiles are consistent with T-lineage ALL.

Effects of intact ricin IT and 4-HC on fresh T lineage ALL blast progenitors. Table 3 shows the efficacy of the clinical Minnesota and Baltimore purging protocols against leukemic bone marrow progenitor cells freshly obtained from T-lineage ALL patients. Treatment with an equimolar cocktail of T101-R and G3.7-R at 1 μ g/mL (4.8 nmol/L) resulted in a significant inhibition of T-lineage blast colony formation in all 12 cases, whereas a mixture of free T101, G3.7 mAb (0.75 μ g/mL = 5 nmol/L), and unconjugated ricin (0.30 μ g/mL = 5 nmol/L) did not elicit a significant inhibition of blast progenitor cells. Control IT GK1.5-R did not affect the colony-forming ability of T-lineage ALL progenitor cells (Table 3). Furthermore, [T101-R + G3.7-R] IT-cocktail only minimally inhibited normal myeloid (ie, CFU-GM), erythroid (BFU-E, CFU-E), or early multilineage (CFU-GEMM) bone marrow progenitor cells at 1 μ g/mL (data not shown). These findings illustrate the selectivity and cell-type specificity of IT-mediated inhibition of T-lineage blast progenitors. The inhibition of leukemic progenitor cells ranged from 0.7 log (=79.7%) to >3.0 logs (=99.9%). However, purging was not complete, and residual T-lineage blast colonies were observed after this ex vivo marrow treatment regimen in eight of 12 cases (UPNs 1, 4-10). 4-HC was also a highly potent inhibitor of leukemic T-lineage progenitor cells. The inhibition of T-lineage blast progenitors after treatment with 100 μ g/mL (=341 μ mol/L) 4-HC ranged from 0.5 log (=64.7%) to 2.5 logs (=99.8%). However, purging was not complete, and residual blast colonies grew in six of nine cases (UPN 1, 4, 6, 8, 10, and 12). By comparison, in two independent experiments, 100 μ g/mL 4-HC completely inhibited CFU-GM, BFU-E, and CFU-GEMM from normal marrow samples (not shown).

A combination of [T101-R + G3.7-R] IT-cocktail (1 μ g/mL) and 4-HC (100 μ g/mL) was effective against leukemic progenitor cells from T-lineage ALL patients, and no residual blast colonies were observed after this treatment protocol

Table 2. Colony Growth and Immunological Features of Colony Blasts

UPN	Blasts Colonies/ 10 ⁵ BM MNC	PE (%)	Average Colony Size (cells)	Immunophenotype of Colony Blasts (%) ^a							
				Single Color IF				Dual Color IF			
				TdT ⁺	CD2 ⁺	CD5 ⁺	CD7 ⁺	TdT ⁺ & CD5,7 ⁺	TdT ⁺ & CD5,7 ⁺	TdT ⁺ & CD5,7 ⁺	TdT ⁺ & CD5,7 ⁺
1	308 (260, 356)	0.308	200-500	100	5	86	84	95	0	2	3
2	52 (50, 53)	0.052	200-500	ND	ND	ND	ND	81	0	7	12
3	54 (48, 60)	0.054	50-200	80	49	21	84	75	0	19	6
4	703 (691, 715)	0.703	100-500	ND	80	74	60	75	0	10	15
5	1546 (1381, 1711)	1.546	50-100	100	90	94	86	93	7	0	0
6	1099 (991, 1207)	1.099	50-500	95	41	78	52	ND	ND	ND	ND
7	49 (41, 57)	0.049	200-500	ND	84	98	72	75	6	3	16
8	399 (350, 448)	0.399	100-200	ND	75	85	70	93	0	3	4
9	331 (325, 336)	0.331	100-200	100	85	78	95	91	0	3	6
10	227 (210, 244)	0.227	200-500	ND	95	75	74	85	10	0	5
11	56 (47, 65)	0.056	200-500	ND	69	75	84	78	18	1	3
12	1045 (1002, 1088)	1.045	50-200	100	99	95	24	100	0	0	0

Abbreviations: MNC, mononuclear cells; PE, plating efficiency; ND, not determined.

^aThe immunological marker profiles of day 7 colony blasts were evaluated in cytospin preparations by indirect IF as described.⁹ Quantitative data were obtained from examination of 100-200 cells. Results are expressed as percentage (%) of immunofluorescent cells.

Table 3. Effects of Intact Ricin IT and 4-HC Treatment on Fresh T-Lineage Leukemic Marrow Progenitor Cells From T-Lineage ALL Patients

UPN	No Treatment	Mean Number of T-Lineage Blast Colonies/10 ⁵ BM MNC						Log Kill of T-Lineage Leukemic Progenitor Cells*					
		T101mAb + G3.7mAb			T101-R + GK1.5-R			T101-R + G3.7mAb			T101-R + GK1.5-R		
		R	GK1.5-R	G3.7-R	4-HC	4-HC	4-HC	R	GK1.5-R	G3.7-R	4-HC	4-HC	4-HC
1	308	187	354	1	0.5	1	0	0.2	0.0	2.5	2.5	2.5	>2.5
2	52	37	55	0	ND	ND	ND	0.1	0.0	>1.7	ND	ND	ND
3	54	ND	ND	0	0	ND	0	ND	ND	>1.7	>1.7	ND	>1.7
4	703	452	ND	57	9	ND	0	0.2	ND	1.1	1.9	ND	>2.8
5	1546	957	1325	19	ND	ND	ND	0.2	0.1	1.9	ND	ND	ND
6	1099	1217	1025	3	53	35	0	0.0	0.0	2.6	1.3	1.5	>3.0
7	49	ND	ND	4	0	ND	0	ND	ND	1.1	>1.7	ND	>1.7
8	399	274	351	4	141	107	0	0.2	0.1	2.0	0.5	0.6	>2.6
9	331	296	350	2.5	ND	ND	ND	0.0	0.0	2.1	ND	ND	ND
10	227	ND	ND	46	48	ND	0	ND	ND	0.7	0.7	ND	>2.4
11	56	ND	73	0	0	0	0	ND	0.0	>1.7	>1.7	>1.7	>1.7
12	1045	973	1100	0	123	90	0	0.0	0.0	>3.0	0.9	1.1	>3.0

Abbreviations: MNC, mononuclear cells; ND, not determined.

*Fresh T-lineage ALL blasts were cultured after various treatment regimens for seven days and assayed for colony formation as described in Materials and Methods. IT were used at 1 μ g/mL and 4-HC at 100 μ g/mL. Log Kill was calculated as Log (PE of sham-treated blasts)/(PE of treated blasts).

in any of the eight cases studied. In five of these eight cases (UPNs 1, 4, 6, 8, and 10), residual blast colonies were observed after treatment with [T101-R + G3.7-R] or 4-HC alone. In none of the eight cases was the combined protocol less effective than IT or 4-HC alone. The additional killing was specific and not due to some unexpected interactions between ricin IT and 4-HC, since irrelevant IT GK1.5-R only minimally affected the cytotoxicity of 4-HC against fresh clonogenic T-lineage ALL blasts (Table 3). As with 100 μ g/mL 4-HC alone, no in vitro hematopoietic colonies (ie, no CFU-GM-, BFU-E-, CFU-E, or CFU-GEMM-derived colonies) were obtained after treatment of normal marrow samples with this combination protocol in any of three independent experiments (data not shown). It may not be necessary to use 4-HC at as high as 100 μ g/mL if it is combined with IT. In four patients (UPN 1, 4, 6, and 8), no residual T-lineage blast colonies were observed after treatment with a ten-fold lower concentration of 4-HC (10 μ g/mL) and [T101-R + G3.7-R] (1 μ g/mL), a purging protocol that only minimally affected normal monopotent (CFU-GM, BFU-E, and CFU-E) or multilineage (CFU-GEMM) marrow progenitor cells (data not shown).

In summary, we have compared the antileukemic efficacy of clinical marrow purging protocols employing IT and 4-HC for autologous BMT in T-lineage ALL at the level of leukemic T-lineage marrow progenitor cells, using fresh marrow samples from 12 T-lineage ALL patients. These studies are of special interest because, to our knowledge, they represent the first analysis of the antileukemic efficacy of clinical purging protocols against fresh clonogenic ALL blasts. Two major findings are that (1) leukemic marrow progenitor cells from T-lineage ALL patients were quite sensitive to the lethal actions of ricin and 4-HC, and (2) an intact ricin IT-cocktail directed against CD5 and CD7 surface determinants, as well as 4-HC, could efficiently kill

T-lineage blast progenitors. Nevertheless, our findings also indicate that these purging protocols may be suboptimal, and combined immunochemotherapy may be a more effective purging strategy in autologous BMT for T-lineage ALL. These data are consistent with and further extend the published experience on the clinical potential of combined purging protocols for autologous BMT in ALL.^{7,11,13,14} The leukemic T-lineage progenitor cell assay described in this paper provides a basis for future comparative studies of different clinical purging protocols. Despite its obvious advantages over clonogenic assays which employ homogeneous cell lines, this colony assay is limited in its sensitivity because of the low plating efficiency of fresh leukemic progenitor cells. Nevertheless, we were able to compare the efficacy of three distinct purging protocols at a sensitivity level of 3 logs. Further improvement of the sensitivity may be achieved by defining better in vitro growth conditions for T-lineage ALL blasts.

In view of recent clinical data showing that posttransplant leukemic relapse remains a major unsolved problem in both allogeneic and autologous BMT for ALL,¹ we conclude that future autologous BMT studies in ALL should focus on designing more effective cytoreductive preparative regimens, and application of combined treatment protocols such as IT + 4-HC for more effective purging of autografts should be considered. A clinical phase I trial would be required to verify the repopulation capacity of autografts purged ex vivo with IT + 4-HC, since the toxicity to repopulating pluripotent human bone marrow stem cells cannot be measured by currently available in vitro cell culture techniques.

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ANNOUNCEMENTS

INTERNATIONAL SOCIETY OF HEMATOLOGY (ISHPAD) VI INTERNATIONAL CONGRESS OF THE ASIA-PACIFIC DIVISION

**December 7-10, 1987
Bombay, India**

The Organizing Committee of the VI Congress has assigned Mr. Aloke C. Bagchi to function as the Official Liaison and Program Coordinator for the United States and Canada for the promotion and dissemination of Congress information and the handling of all special travel programs for the delegates and their spouses from North America.

All ASH members are welcome to contact Mr. Bagchi at 6101 N Talman Ave, Chicago, IL 60659-0557, telephone (312) 465-8337, for the Congress brochure, registration forms, and information pertaining to the Congress and the special economy program for India and Nepal.

BAYLOR UNIVERSITY MEDICAL CENTER, DALLAS, TEXAS "ONCOLOGY PRACTICE 1987: A PERSPECTIVE" Snowmass, Colorado February 1-7, 1987

For further information, contact: Stephen E. Jones, MD, Sammons Cancer Center #4800, Baylor University Medical Center, 3500 Gaston Avenue, Dallas, TX 75246

AMERICAN BOARD OF INTERNAL MEDICINE 1987 SUBSPECIALTY EXAMINATIONS IN MEDICAL ONCOLOGY AND CRITICAL CARE MEDICINE

Registration Period: January 1, 1987 through April 1, 1987
Examination Date: November 10, 1987

For information and applications forms, please contact: Registration Section, American Board of Internal Medicine, 3624 Market Street, Philadelphia, PA 19104.

CLINICAL HEMATOLOGY AND ONCOLOGY: 1987

**February 16-18, 1987
Sponsored by Scripps Clinic and Research Foundation, San Diego, CA
Laurence Harker, MD, Program Chairman**

This conference is designed for hematologists, oncologists, internists, pediatricians, and pathologists interested in the diagnosis and management of hematologic and oncologic disorders. An outstanding faculty from across the country has been assembled to discuss various aspects of these fields, to aid clinicians in sorting out the important from the trivial, and to help identify those recent advances which will withstand the test of time. 21 hours Cat. I.

For further information and application forms, contact: Bonny Mower, Department of Academic Affairs, Box 400S, Scripps Clinic and Research Foundation, 10666 N Torrey Pines Rd, La Jolla, CA 92037. Telephone (619) 457-8556.

AMERICAN SOCIETY OF HEMATOLOGY

Spring Symposium on Hematopoietic Growth Factors

Durham, North Carolina

April 20–21, 1987

The American Society of Hematology is holding a two-day symposium on hematopoietic growth factors sponsored by Burroughs Wellcome Company, to be held at the Searle Center in Durham, North Carolina on April 20 and 21, 1987. The topics will include the molecular biology of the hematopoietic growth factors, their biological actions, and the results of in vivo studies with these molecules in experimental animals and humans. Among the participants will be Steven Clark, Malcolm Moore, David Golde, Judith Gasson, Ken Kaushansky, Gordon Wong, Peter Quesenberry, John Adamson, and Colin Sieff.

Additional information and details of the program are available through Dr Wendell F. Rosse, PO Box 3934, Duke University Medical Center, Durham, NC 27710. Individuals interested in submitting abstracts or poster presentations, or preregistering are encouraged to do so by March 1, 1987. Maximum registration: 200.

ERRATUM

In the article by Griesser et al entitled "The Structure of the T Cell Gamma Chain Gene in Lymphoproliferative Disorders and Lymphoma Cell Lines" which appeared in the August 1986 issue (Volume 68, pp 592–594), two errors were made. In the abstract, the sentence "TcR γ was rearranged in all T cell lymphomas and B cell lymphoma cell lines" should read "TcR γ was rearranged in all T cell lymphomas and in some B cell lymphomas and B cell lymphoma cell lines." The last sentence in the introduction should read: "These studies indicate that rearrangement of TcR γ occurs before TcR β and that rearrangement of the γ chain locus by itself is a poor indicator of cell lineage."

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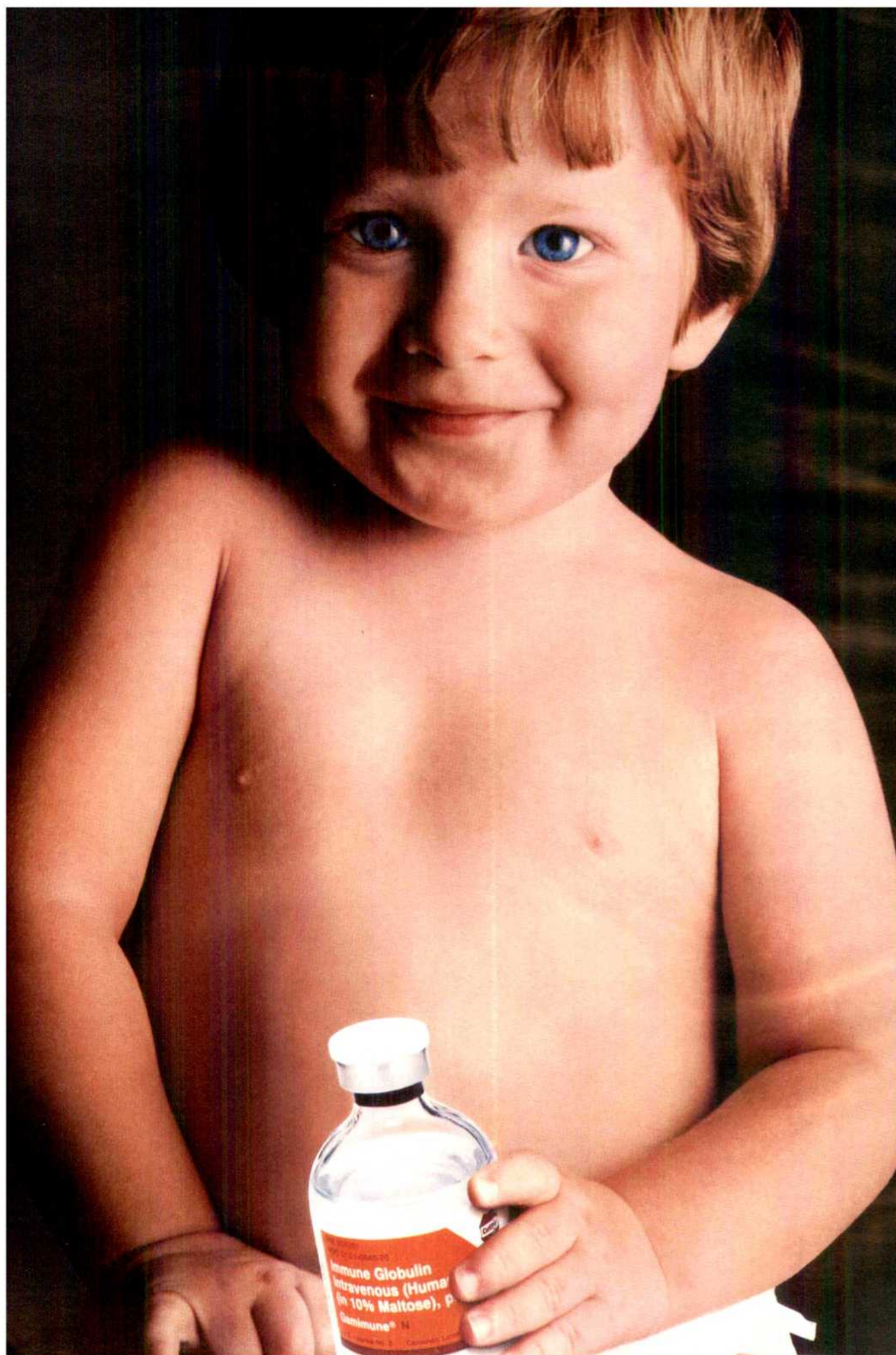
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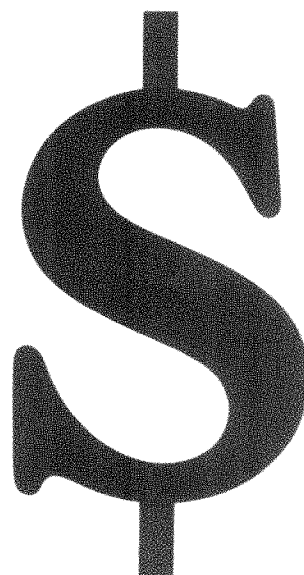
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The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood, there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune[®] N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune[®] N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune[®] N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune[®] N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune[®] N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune[®] N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

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In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PL^{A1} alloimmune antibody with platelet anti PL^{A1} specificity) also responded to treatment with Gamimune[®] N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N should be considered, in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune[®] N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune[®] N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune[®] N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune[®] N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune[®] N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune[®] N may be diluted with 5% dextrose in water (D5W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune[®] N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune[®] N. It is not known whether Gamimune[®] N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune[®] N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune[®] N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune[®] N. Symptoms reported with Gamimune[®] N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N at the infusion site was reported in some cases.

In further studies of Gamimune[®] N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune[®] N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune[®] N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune[®] N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune[®] N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune[®] N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune[®] N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune[®] N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune[®] N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune[®] N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes, if well tolerated, the rate may be gradually increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. GAMIMUNE[®] N Package Insert. 2. Mitra G, Wong MF, Mosen MM, et al: Elimination of infectious retrovirus during preparation of immunoglobulins. *Transfusion* 1986;26(4): 394-397.

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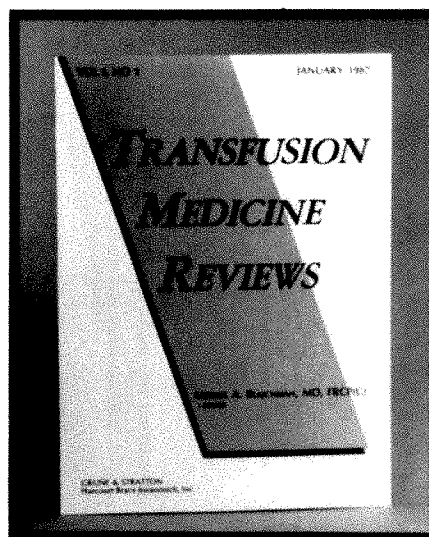
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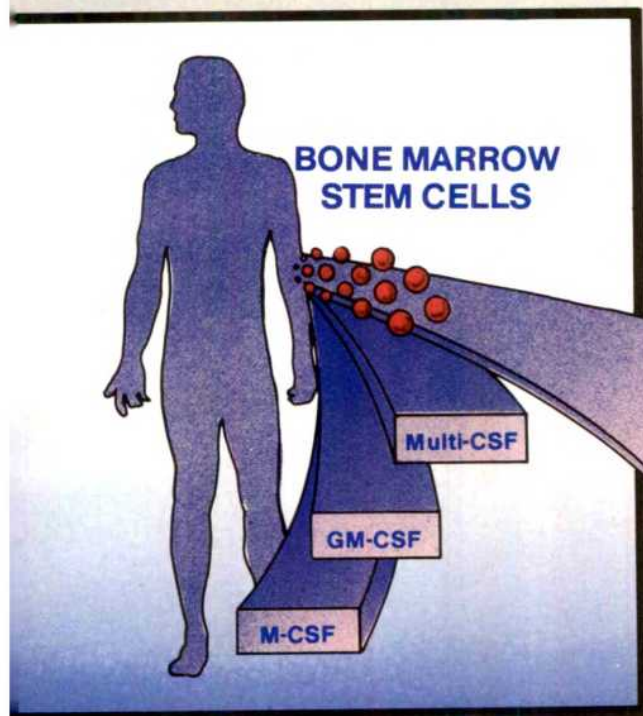
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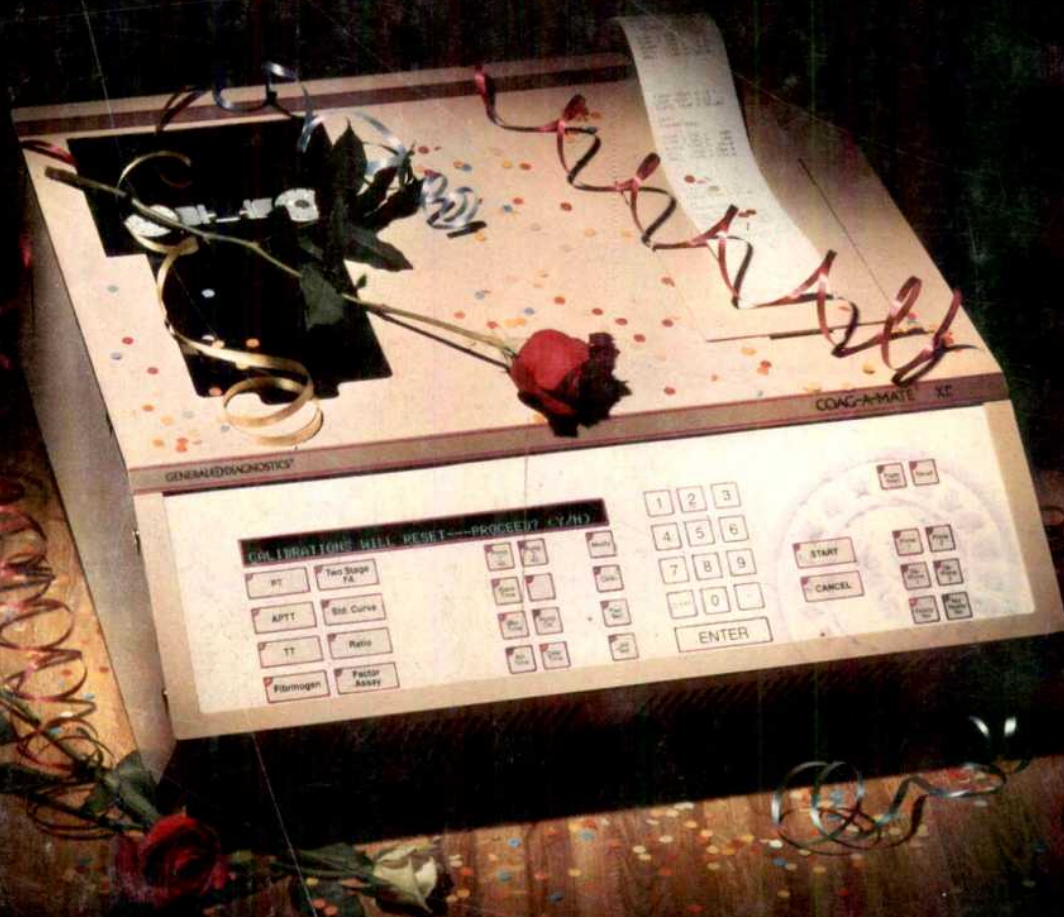


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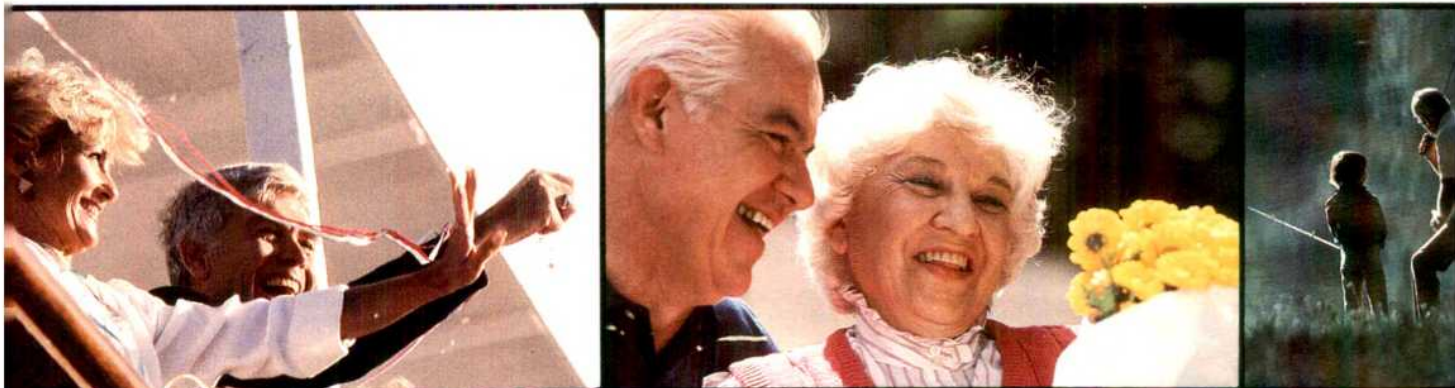
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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B₁₂. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below 5×10^{-8} M.^{1,2} The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.^{3,4,5}

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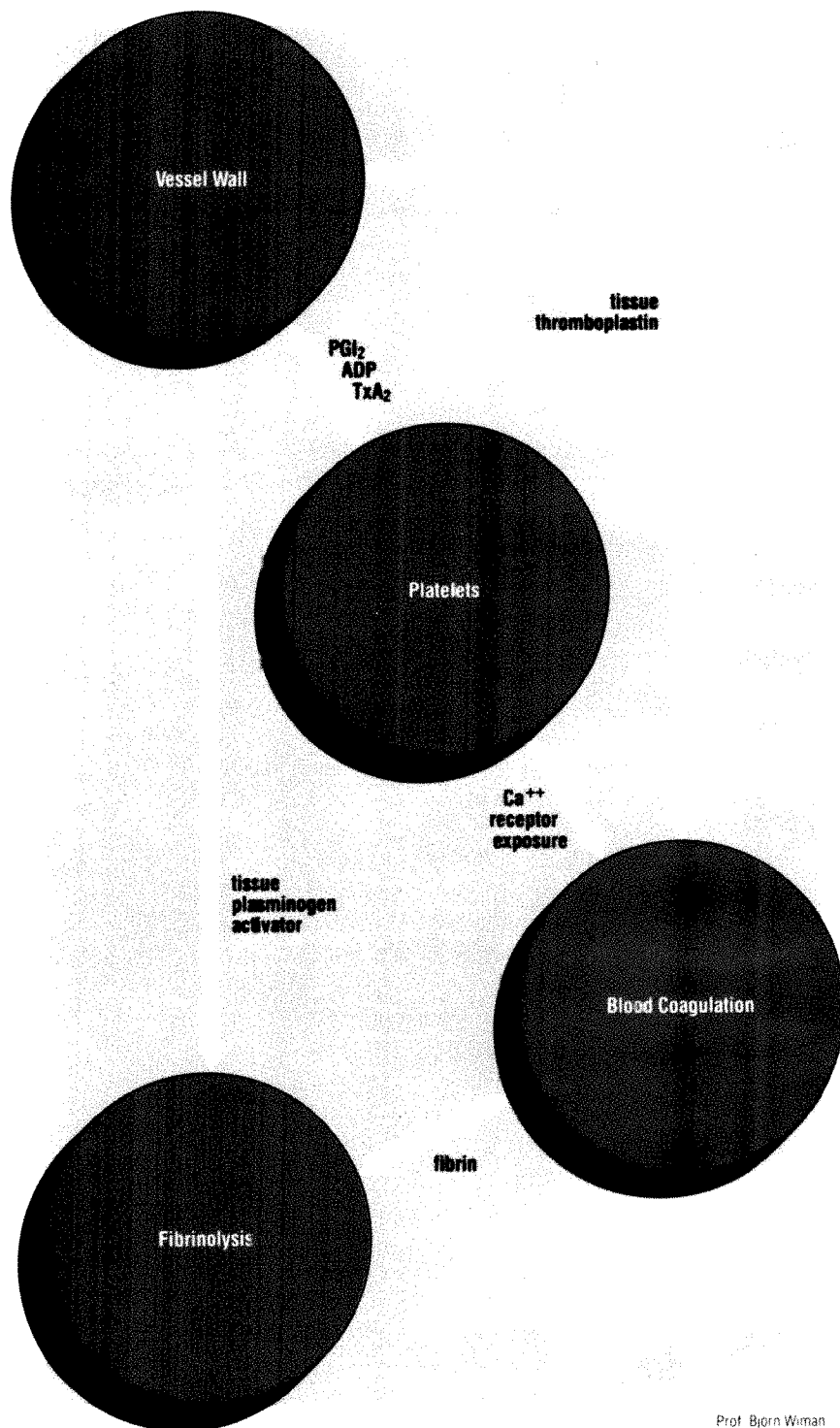
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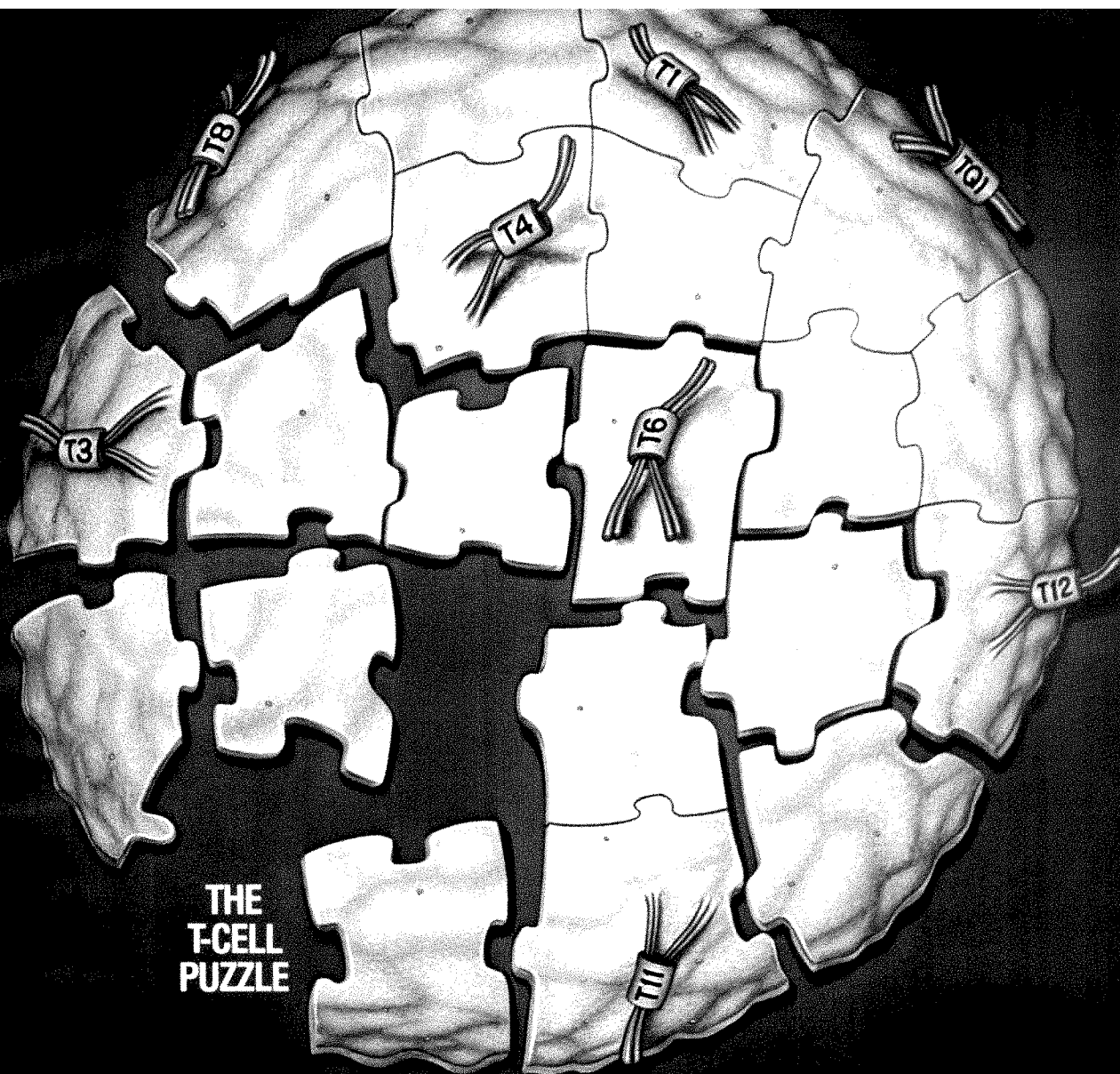
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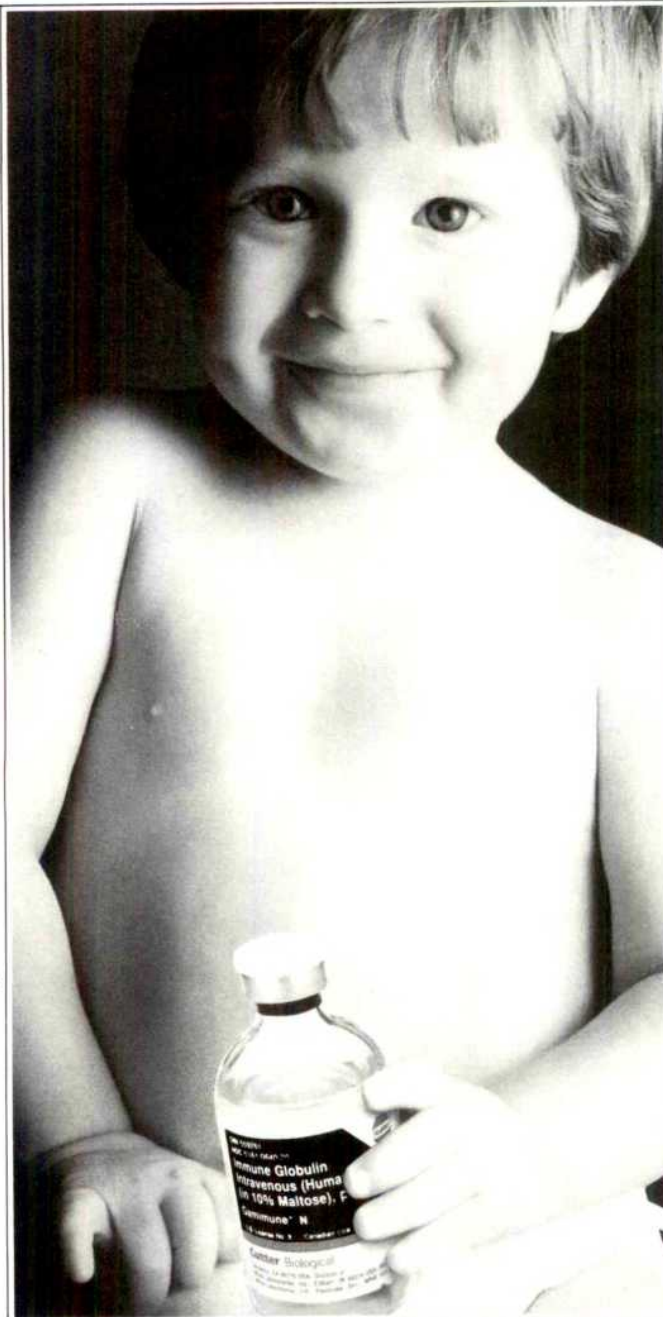
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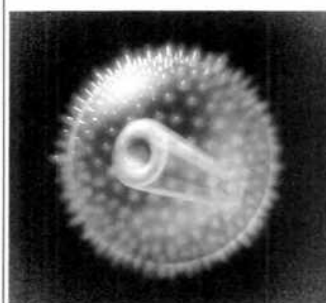


Documented in vitro inactivation of HTLV-III virus[†]

[†]The Cohn-Oncley process used to produce Gamimune®N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune®N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 TCID (Tissue Culture Infectious Doses) reduction of HTLV-III.[‡]

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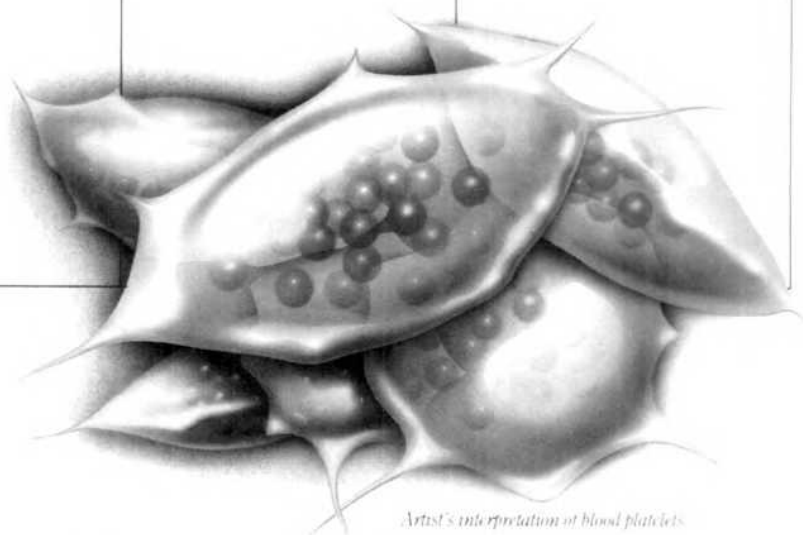
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Artist's interpretation of HTLV-III virus.

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Artist's interpretation of blood platelets.

*It is not possible to predict which patients with ITP will respond to therapy.

¹In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

GAMIMUNE[®] N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gammimune[®] N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gammimune[®] N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The *in vivo* half-life of Gammimune[®] N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood, there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gammimune[®] N is 16.5 mEq/L (~0.3 mEq/g protein), a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gammimune[®] N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gammimune[®] N at a dose of 150 mg/kg body weight, following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gammimune[®] N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gammimune[®] N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gammimune[®] N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gammimune[®] N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gammimune[®] N may initiate a therapeutic increase in the platelet count. In clinical studies of Gammimune[®] N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gammimune[®] N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gammimune[®] N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gammimune[®] N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gammimune[®] N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gammimune[®] N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PLA² alloimmune antibody with platelet anti PLA¹ specificity) also responded to treatment with Gammimune[®] N, at a dose of 400 mg/kg body weight daily for 5 days, there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gammimune[®] N should be considered, in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gammimune[®] N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gammimune[®] N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gammimune[®] N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gammimune[®] N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gammimune[®] N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gammimune[®] N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gammimune[®] N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gammimune[®] N. It is not known whether Gammimune[®] N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gammimune[®] N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gammimune[®] N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gammimune[®] N. Symptoms reported with Gammimune[®] N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gammimune[®] N at the infusion site was reported in some cases.

In further studies of Gammimune[®] N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gammimune[®] N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gammimune[®] N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gammimune[®] N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gammimune[®] N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gammimune[®] N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gammimune[®] N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gammimune[®] N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gammimune[®] N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gammimune[®] N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes, if well tolerated, the rate may be gradually increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. GAMMIMUNE[®] N Product License Application. 2. Lundblad JL, Mitra G, Steinberg MM, et al. Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(Jul-Aug): S382-S390. 3. Mitra G, Wong MF, Mozen MM, et al. Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26(4):394-397.

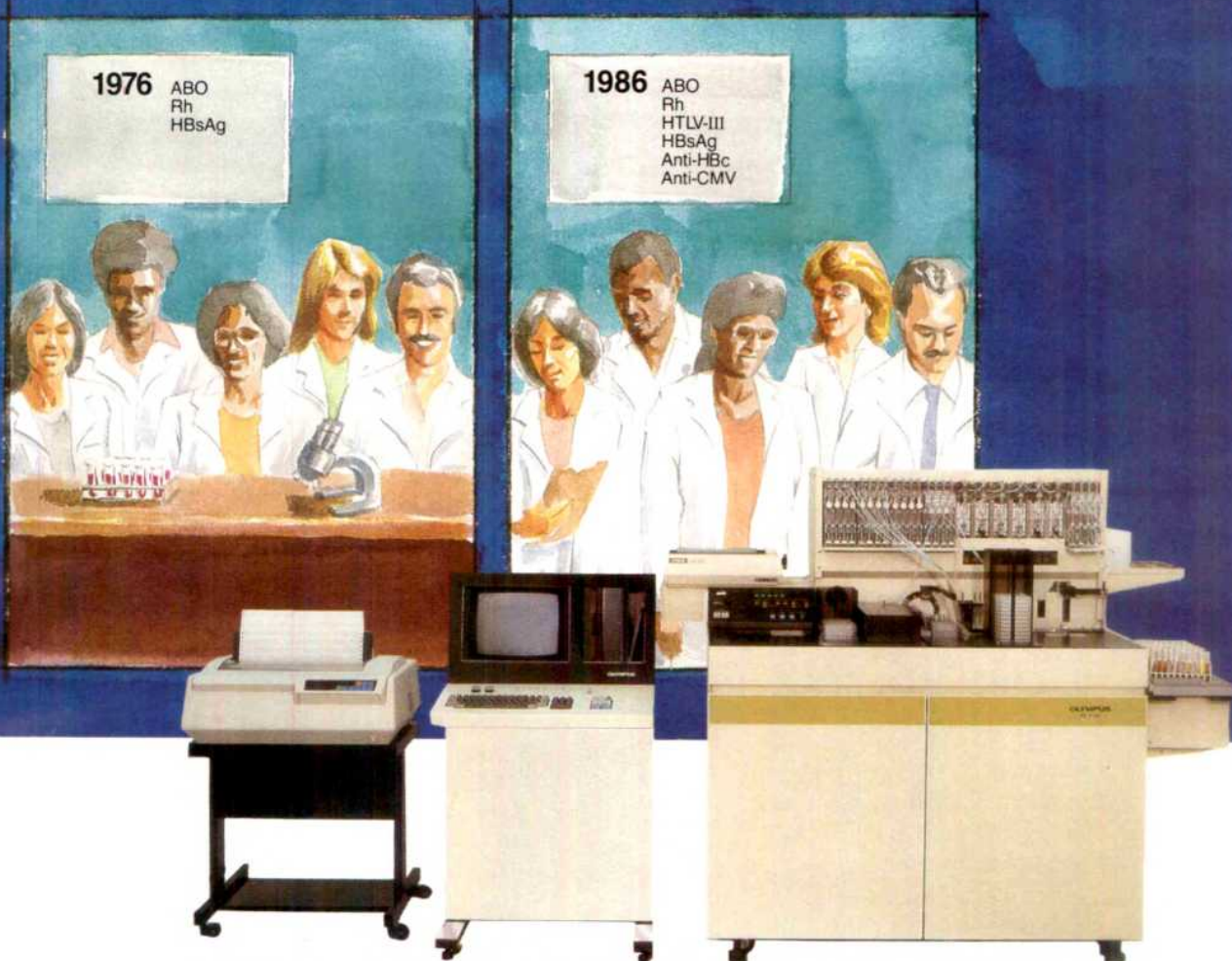
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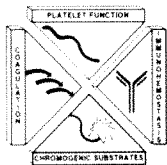
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When and Where?

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May	1	Houston, TX		5	Washington, DC
May	18	Minneapolis, MN			
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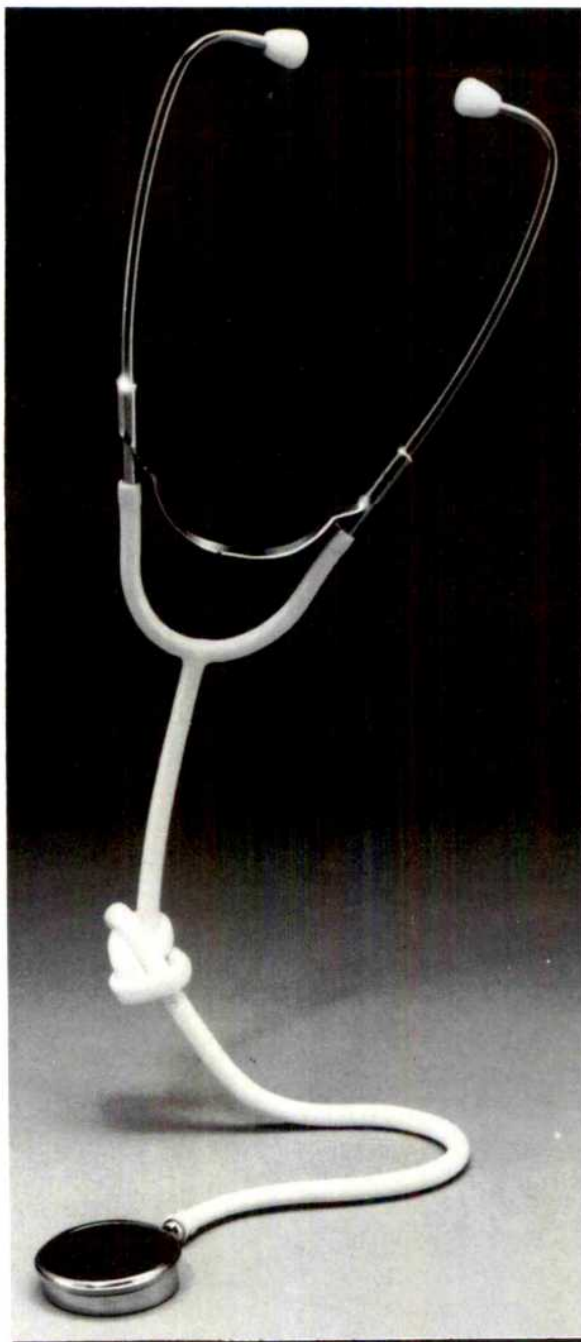
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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. *Br J Haematol* 38:161, 1978

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2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. *Semin Psychiatry* (in press)

Complete book:

3. Lillie RD: *Histopathologic Technic and Practical Histochemistry* (ed 3). New York, Blakiston, 1965, p 39

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4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): *Hemic Cells In Vitro*, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): *Gamma Globulins, Proceedings of the Third Nobel Symposium*. New York, Wiley, 1967

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6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. *Clin Res* 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. *Blood* 47:879, 1976 (letter)

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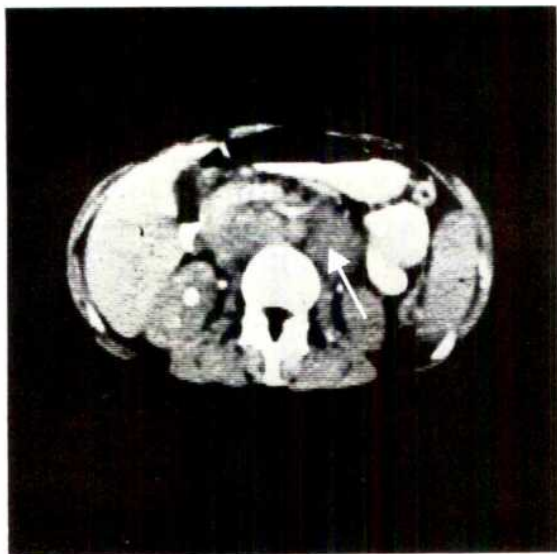
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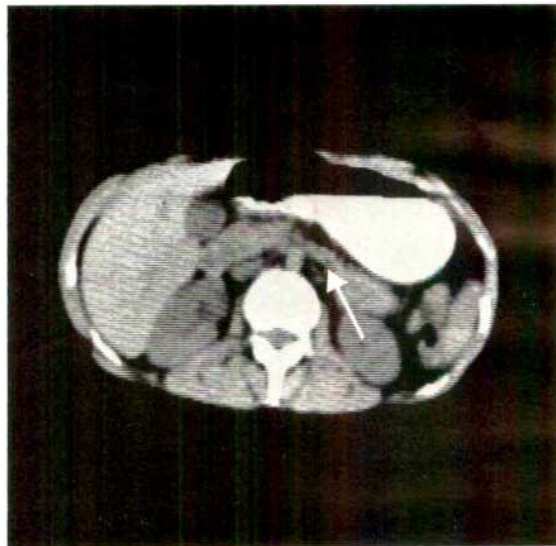
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Abdominal/Pelvic CT
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No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al*: Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al*: Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al*: Combination chemotherapy advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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M-BACOD

4. Skarin AT, Canellos GP, Rosenthal DS, et al: Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
5. Anderson KC, Skarin AT, Rosenthal DS, et al: Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, et al: Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), m-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

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Brief Summary of Prescribing Information (1) 10 84 For complete prescribing information please consult product literature

WARNING

It is recommended that Blenoxane[®] be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma

Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

- (1) Maintain FiO_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.
- (2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinoma treated with a combination of Blenoxane and Velban[®]. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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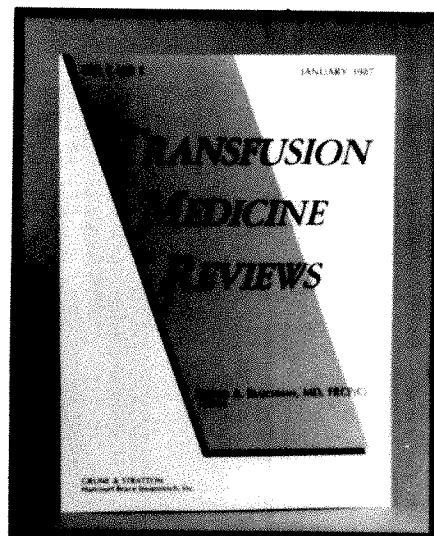
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- The association of blood group antigens with red cell membrane glycoproteins. *David J. Anstee*
- The effects of red cell antigens and blood transfusion on renal transplantation. *Morris A. Blajchman*
- The role of the hematocrit in bleeding. *Bernard Boneu (with F. Fernandez)*
- Complications associated with apheresis procedures. *Marc Boogaerts*
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- The significance of reticuloendothelial function in the immune destruction of blood cells. *John Kelton*
- Immunogenicity of platelet membrane glycoproteins. *Tom Kunicki*
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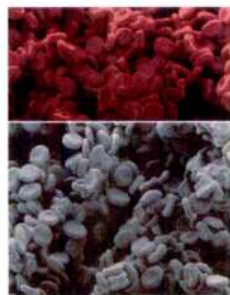
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REVIEW

The Kinetics of Immune Reconstitution After Human Marrow Transplantation

By Lawrence G. Lum

Human marrow transplantation for the treatment of malignant and nonmalignant disorders is becoming an established modality of therapy. As in any aggressive therapeutic modality, the benefits must be balanced with the risks of the therapy. The aggressive chemoradiotherapy used to prepare patients for marrow transplantation creates a transient immunodeficiency disorder postgrafting until the transferred donor marrow reestablishes a competent immune system. Immune reconstitution posttransplant follows a general pattern developing from immature to mature immune functions. Immune reactivity during the first month postgrafting is extremely low. Cytotoxic and phagocytic functions recover by day 100, while more

specialized and cooperative functions of T and B cells remain impaired up to one year or more postgrafting. After the first year postgrafting, the various components of the immune systems of most healthy marrow recipients begin to work synchronously, whereas the immune systems of recipients with chronic graft-v-host disease (GVHD) remain crippled. Recent evidence shows that transfer of specific immunity from marrow donors to marrow recipients plays a role in reestablishing immunocompetence. Transferred antigen-specific immunity may explain why more recipients do not die from overwhelming infections.
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BONE MARROW transplantation is an accepted mode of therapy for immunodeficiency disorders, aplastic anemia, or hematologic malignancies.¹⁻² The chemoradiotherapy used to prepare marrow graft recipients ablates existing normal and abnormal immunohematopoietic marrow elements, provides immunosuppression to permit engraftment, and creates space in the marrow microenvironment for the donor marrow to develop. Although the immunodeficiency is created under carefully controlled circumstances, the marrow graft recipients are extremely susceptible to life-threatening infections with a variety of bacterial, viral, and fungal pathogens.³⁻⁶ The first 100 days postgrafting is the interval of maximum risk; however, marrow graft recipients with chronic graft-v-host disease (GVHD) continue to be at risk for bacterial, viral, and, less frequently, fungal infections.

Marrow transplantation offers a model of iatrogenic immunodeficiency that is unique in that immunity generally recovers if the transplant recipient does not succumb to transplant related complications or recurrent disease. This model permits dissection of the various elements of the human immune system as they reappear under the influence of various treatment modalities postgrafting. An understanding of the kinetics of immune reconstitution in the marrow transplant model will enhance our understanding of immunodeficiency disorders. Parallels can be drawn between the transplant model and the defects seen in immunodeficiency disorders and the regressing immune systems of patients with acquired immunodeficiency syndrome (AIDS).

The various in vivo and in vitro immune function tests of the marrow recipients will be discussed in terms of the first 100 days (short-term recipients) and from 100 days to years (long-term recipients) postgrafting. The areas that will be

reviewed include: (1) general considerations of immunity, (2) issues related to acute GVHD in short-term recipients, (3) issues related to chronic GVHD in long-term recipients, (4) mucosal defense system, (5) recovery of granulocyte and accessory cell functions, (6) recovery of cytotoxic functions of lymphocytes, (7) T cell-mediated immunity early postgrafting, (8) T cell-mediated immunity in long-term recipients, (9) B cell-mediated immunity early postgrafting, (10) B cell-mediated immunity in long-term recipients, (11) use of immunomodulators to accelerate immune reconstitution, (12) reimmunizations for marrow recipients, (13) recapitulation of ontogeny, and (14) future directions.

GENERAL CONSIDERATIONS OF IMMUNITY

Studies at a number of transplant centers have shown that the repopulation of the immune and hematopoietic systems is dependent on appropriate proliferation, maturation, and differentiation of donor cells. The signals that direct immune

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reconstitution are largely unknown. However, the key factor in immune reconstruction is time after grafting.⁷⁻¹³ Successful immune reconstruction is dependent on the orchestrated emergence of many different components of the immune system. All marrow graft recipients have a profound impairment of most immune functions during the first four to five months postgrafting, regardless of the type of graft (autologous, syngeneic, or allogeneic), the type of underlying disease, the conditioning regimen, the type of postgrafting immunosuppression, the preparative regimen, or the presence of acute GVHD.⁷⁻¹⁶ Immune reconstitution in most healthy long-term survivors is successfully completed by one to two years postgrafting, whereas the same process in recipients with chronic GVHD is delayed, leaving them susceptible to infections.

The marrow recipient progresses from an immunologic state characterized by primitive cytotoxic systems such as natural killer activity, and the predominance of cells with suppressive functions to a more mature immunologic state characterized by finely tuned immune responses. Initially there is reestablishment of phagocytic functions followed by repopulation of the early lymphoid elements responsible first for primitive and then for more specific cytotoxic functions. Subsequently T cells responsible for regulatory functions emerge. The early suppressive T cell signals are followed by the development of mature helper T cell signals. Evidence suggests that the transition from a predominance of suppression to a predominance of helper activity in the recipient can be disrupted by chronic GVHD or its treatment. Transfer of existing donor immunity may play a role in achieving antigen-specific immune reconstitution in both the T and B cell compartments of the recipient immune system.

The role of the host thymus in this reconstitution process remains elusive in human marrow transplantation. Age of recipient at transplant, irradiation, and chemotherapy could all affect the functional capacity of the thymic microenvironment to properly facilitate the maturation of donor T cells. Furthermore, other nonthymic components of the immune system that may play roles in controlling the recapitulation of immune ontogeny could also have been damaged during the preparative regimen. Though ill defined, both thymic and nonthymic factors influence immune reconstitution.

Although host immunity is severely compromised by the preparative regimens, it is not absent. Graft rejections or lack of sustained engraftment in patients with severe aplastic anemia or patients who receive T cell-depleted marrow are probably mediated by persistent host immunity. Pretransplant transfusion sensitization increases graft rejections in patients with aplastic anemia,¹⁶ whereas untransfused aplastic anemia patients have a lower incidence of graft rejection than their transfused counterparts.¹⁷

Graft failures in recipients who receive T cell-depleted bone marrows to prevent acute GVHD suggest that removal of all T cells may be a two-edged sword. Vigorous removal of T cells has decreased the incidence of acute GVHD but has increased the incidence of graft failure and recurrent leukemia. Graft failure occurs presumably due to residual irradiation-resistant host cells that destroy the marrow graft. (The role of cytotoxic T cells in GVHD and host defense is

reviewed on later pages.¹⁸) The general consensus is that certain T cell populations in the marrow inoculum may be needed to facilitate sustained engraftment in the face of host resistance and to maintain immune surveillance against the expansion of residual leukemic clones.

Low levels of persistent host immunity may play a protective role against bacterial, fungal, and viral pathogens in the first three months after marrow grafting. Persistent host-type isohemagglutinins can be detected up to three to four months postgrafting in recipients who receive ABO incompatible marrows.¹³ These findings suggest that surviving chemoradioresistant host lymphocytes or persistent antigen may have a role in the education of naive donor cells.

ISSUES RELATED TO ACUTE GVHD IN SHORT-TERM RECIPIENTS

When the emerging immune system in the recipient does not recognize antigens on host tissues as self, it may mount an appropriate immunologic reaction against foreign antigens (acute GVHD). This ability to attack host tissues demonstrates "misdirected" immunocompetence of the graft. This misdirected immunocompetence may be due to improper thymic reeducation, altered thymic microenvironment, or host-recipient histoincompatibility. Unfortunately, this attack often has very serious effects on the marrow recipient. The principal target organs of acute GVHD in animals and man are the skin, liver, and gastrointestinal (GI) tract.^{1,19-21} Cytotoxic cells are thought to play a major role in the immunologic attack. The clinical manifestations of acute GVHD range from mild skin rashes, impairment of liver function, and GI disturbances such as nausea, vomiting, and diarrhea to severe disease with full thickness skin eruptions with bullae formation, liver failure, bloody diarrhea from denuded intestinal epithelium, and depressed immune functions leading to life-threatening sepsis. Roughly half the marrow recipients transplanted with allogeneic bone marrow from HLA-identical siblings develop acute GVHD. Approximately 10% to 15% of all marrow recipients die of acute GVHD or its associated complications. Acute GVHD, *per se*, results in immunosuppression.

Acute GVHD may be enhanced by the presence of enterobacteria in the gut. The enterobacteria invading the gut mucosa may share antigenic determinants with host epithelium resulting in enhanced cytotoxic immune responses directed at the mucosal lining.²² Observations that support this hypothesis are (1) recipients with acute GVHD have more infections than those without GVHD^{2,23}; and (2) the protective environments of laminar air flow rooms reduce the number of infections and delay the onset of acute GVHD in recipients transplanted for aplastic anemia.²⁴

Since acute GVHD is a major impediment to successful marrow transplantation, immunosuppressive agents have been given pre- or postgrafting to prevent the development of acute GVHD.²³ These agents may further immunosuppress the recipient's immune system and prolong the susceptibility to infections. Methotrexate, cyclophosphamide, and cyclosporine (Cs) have been commonly used to prevent GVHD in man.^{23,25-27} The advantage of Cs over methotrexate is faster

engraftment, less mucositis, decreased platelet support, and a shorter hospital stay. However, immune function tests in those who received Cs prophylaxis for GVHD were not different from those who received methotrexate.²⁸ The recent combination of methotrexate and Cs to prevent GVHD has been more successful than methotrexate alone in reducing the incidence of acute GVHD and in improving long-term survival.²⁹

Adapting the well-characterized murine transplant models using T cell depletion for preventing GVHD in man has been cumbersome at best. Since cytotoxic T cells are thought to mediate the damage to tissues in acute GVHD, methods to remove T cells from marrow inocula have been used to prevent GVHD. These methods include treatment with antithymocyte globulin, monoclonal antibodies (MoAbs) directed at T cells, lectin agglutinin sedimentation, or sheep erythrocyte rosetting.³⁰⁻³⁵ Although T cell depletion procedures have decreased the incidence and severity of acute GVHD, the general consensus is that over-zealous T cell depletion has led to graft failure in recipients transplanted for acute leukemias. Furthermore, vigorous T cell depletion may be removing T cells that mediate a graft-v-leukemia effect. Most transplant groups agree that the incidence of leukemic relapse has increased with T cell depletion of the marrow graft. These procedures may also lead to increased abnormalities of immune reconstitution.

Recent studies show that depleting CD4 cells from the marrow may prevent acute GVHD and still permit sustained engraftment.³⁶ Although most models implicate cytotoxic CD8 cells in the pathogenesis of GVHD, there is no evidence that rules out the participation of CD4 cells or both CD4 and CD8 cells in the pathogenesis of acute GVHD. Follow-up studies on recipients who receive T cell-depleted marrow grafts will help sort out such issues.

No data directly support the hypothesis that specific cytotoxic cells or specific antibodies mediate the immune reaction seen in clinical GVHD.^{2,37,38} Incriminating immunologic evidence for the existence of specific alloreactive T cells or specific antibodies directed at tissues bearing non-HLA antigens is absent.^{38,39} Mixed lymphocyte culture assays using donor-derived cells from marrow recipients with acute GVHD failed to detect proliferative responses to cryopreserved host lymphocytes.⁴⁰ One recent study showed that T cell clones isolated from skin biopsies involved in acute GVHD could proliferate in response to host lymphocytes; however, direct cytotoxicity mediated by such cells was not shown.⁴¹ New approaches for understanding the immunobiology of GVHD are needed. Simple questions remain unanswered, such as "What are the effector and effector cells mediating GVHD in human transplant recipients?" and "What are the target cells in the various organ systems affected by GVHD?"

A promising predictive test for acute GVHD was recently developed.⁴² Donor lymphocytes were sensitized against recipient lymphocytes in vitro and cocultured with recipient skin. The coculture system showed histologic changes similar to acute GVHD and was able to predict acute GVHD. Such a test may be useful in selecting donors for transplantation and selecting the type of GVHD prophylaxis.

ISSUES RELATED TO CHRONIC GVHD IN LONG-TERM RECIPIENTS

Chronic GVHD occurs in 35% to 45% of the patients receiving HLA-identical bone marrow transplants at any time between 100 days and 15 months postgrafting.⁴³ The clinical picture of chronic GVHD resembles autoimmune diseases such as scleroderma, systemic lupus erythematosus, Sjögren's syndrome, lichen planus, and eosinophilic fasciitis. This analogy is supported by the findings of circulating autoantibodies and deposits of immunoglobulin and complement at the dermal-epidermal junction.⁴⁴⁻⁵⁰ The manifestations of chronic GVHD include skin lesions, severe buccal mucositis, keratoconjunctivitis, esophageal and vaginal strictures, large intestinal involvement, chronic liver disease, and failure to thrive.

Increasing patient age at transplant is associated with an increased incidence of chronic GVHD. The presence of nonspecific suppressor cells in the mixed lymphocyte culture assay^{51,52} and the increased suppressor T cell activity in immunoglobulin production assays may reflect similar abnormal nonspecific regulatory phenomena in recipients with chronic GVHD. HLA nonidentity between recipient and marrow donor increased the risk of varicella zoster infections after marrow grafting, and the presence of chronic GVHD predisposed the recipient to late varicella zoster infections.⁵³ The increased incidence of varicella zoster infections may be related to the immunosuppressed state of those with chronic GVHD.

The association between acute and chronic GVHD and their responses to immunosuppressive therapy suggest that chronic GVHD is mediated by lymphocytes reacting against host antigens. In vitro studies support this speculation. Donor-derived lymphocytes from long-term recipients with chronic GVHD exhibited unidirectional mixed lymphocyte culture reactivity to cryopreserved host lymphocytes and cytotoxic activity directed at host fibroblasts.^{54,55} The unidirectional mixed lymphocyte culture reactivity was present in a higher proportion of recipients with chronic GVHD than in long-term recipients without chronic GVHD.⁵⁴ These cells were not detected in healthy recipients presumably due to the presence of specific suppressor T cells that suppress the immune reaction of donor cells to trinitrophenol-modified host cells.⁵⁶ The presence of nonspecific cells to unrelated cells in mixed lymphocyte cultures and increased suppressor T cell activity after pokeweed mitogen activation in half of the recipients with chronic GVHD may be an attempt by the newly developing immune system to compensate for its overactivity directed at non-HLA antigens. Lack of appropriate thymic reeducation of donor lymphocytes by a defective, damaged, or histoincompatible thymic microenvironment may contribute to the pathogenesis of chronic GVHD.

MUCOSAL DEFENSE SYSTEM

Although difficult to quantify, chemoradiotherapy damage to the mucosa in the marrow recipient is a major insult to the host immune system. The mucosal defense systems are then further damaged by acute GVHD. Alterations in the integrity of mucosal linings and the impaired local IgA

secretion by B cells in the linings of the intestinal, respiratory, and urinary tracts may provide easy access for invading pathogens. It is clear from the types of infections that occur and the timing of such infections that mucosal immunity ("the first line of defense") is severely impaired in the first 100 days postgrafting and continues to be impaired in recipients who have chronic GVHD. Skin biopsies from long-term recipients with chronic GVHD show histologic evidence of sialadenitis, stomatitis, or both with sialodochitis.⁵⁷ Salivary gland involvement was associated with decreased or absent levels of IgA, decreased salivary flow rates, and increased concentrations of sodium, albumin, and IgG.⁵⁷ If one adds these findings to the low serum IgG and IgM levels in the first several months after grafting and serum IgA levels that remain low for more than one year postgrafting in the majority of marrow graft recipients,^{7-9,12,13} it is not surprising that fatal infections occur despite protection afforded by antibiotics, laminar air flow rooms, granulocyte transfusions, and immune globulin transfusions.

PHAGOCYTIC AND ACCESSORY CELL FUNCTIONS

Studies on neutrophils show that their numbers normalize early, but they have impaired chemotaxis up to four months postgrafting.⁵⁸⁻⁶⁰ Neutrophil iodination capacity, a measure of the ability of neutrophils to phagocytize and activate oxygen-dependent mechanisms of intracellular killing, were not different from normal controls during the early period.⁵⁸ These findings partially explain why fungal and bacterial infections are common postgrafting when neutrophils are absent and neutrophil chemotaxis is impaired.

Peripheral blood monocytes from marrow recipients can be shown to be of donor origin by cytogenetic markers as early as 41 days postgrafting.⁶¹ Tissue-fixed macrophages in the lung and liver are of donor type by day 80 postgrafting.^{62,63} Monocytes have been reported to exhibit normal chemotaxis, adherence, phagocytosis, and cytotoxicity early postgrafting.⁶⁴

Studies on the antigen-presenting accessory cell functions of peripheral blood monocytes from marrow recipients show that monocytes can successfully present killed *Escherichia coli* bacteria in proliferation assays.⁶¹ Enriched monocyte populations from the majority of short-term and long-term marrow recipients were capable of providing accessory cell function in T cell-dependent pokeweed mitogen-stimulated immunoglobulin production.⁶⁵ Suppressor activity in pokeweed mitogen-stimulated immunoglobulin production mediated by monocytes was detected in only a few marrow recipients with chronic GVHD.⁶⁵ Interleukin 1 (IL 1) production by monocytes from marrow recipients is normal by five to six weeks postgrafting.⁶⁶ By six weeks posttransplant, monocyte functions are generally intact.

CYTOTOXIC FUNCTIONS OF LYMPHOCYTES

Impaired T cell-mediated or natural killer (NK) cell-mediated cytotoxicity directed at viruses may account for nearly one half of the allogeneic recipients developing infections with the herpes group of viruses postgrafting. Cytomegalovirus (CMV) accounts for roughly 60% of the inter-

stitial pneumonias that occur during the first three months postgrafting.⁶ Attempts to prevent or treat CMV pneumonias with various agents or CMV immune globulin have met with variable success.⁶⁷⁻⁷²

The role of NK cells directed at viruses and cytotoxic T cells directed at viruses during the immediate postgrafting period remains controversial. Most studies show that NK activity against K562 or Chang targets, lectin-dependent cellular cytotoxicity, and antibody dependent cellular cytotoxicity recover rapidly to normal levels in the majority of marrow recipients by 30 to 50 days postgrafting,⁷³⁻⁷⁵ but up to 20% of the marrow recipients continue to have deficient cytotoxic functions a year or more postgrafting.⁷³ One study showed that HLA-restricted CMV-specific cytotoxic T lymphocyte responses to CMV and natural killer cell responses to CMV develop postgrafting and that low or nonexistent cytotoxic responses occur at the onset of CMV infections in recipients who develop fatal infections.⁷⁴ Another study showed that NK activity fell from normal levels when recipients developed severe viral infections, and there was a correlation between the incidence of acute GVHD and high NK activity.⁷⁵ Other studies show no significant correlations between cytotoxic activity and clinical findings such as infections, GVHD, and recurrent leukemia.⁷³

Impaired cell-mediated lympholysis (CML) occurred in cells from recipients with acute and chronic GVHD, whereas most short-term and long-term recipients without GVHD had normal CML responses.⁷⁶ Interleukin 2 (IL 2) production was reduced in short-term recipients with acute GVHD.^{76,77} The addition of IL 2 to the mixed lymphocyte cultures during the sensitization phase could restore CML reactivity in lymphocytes from recipients with acute GVHD but not in lymphocytes from recipients with chronic GVHD.⁷⁶ One simple explanation for the heterogeneous and conflicting reports on cytotoxic cell function would be the differences in the techniques used to study recipients and the differences in the study populations. A unifying and biologically relevant explanation is that the current studies may reflect a spectrum of maturational stages of cytotoxic cells.

T CELL-MEDIATED IMMUNITY EARLY POSTGRAFTING

Donor-derived T and B cells repopulate the peripheral blood in allogeneic marrow recipients within the first three months. However, repopulation of T cell subsets as defined by surface phenotypic markers using monoclonal antibodies or Fc-receptor rosetting occurs at different rates for both subsets and individual recipients. The consensus of most studies using phenotypic markers for the helper-inducer phenotype (CD4) and the suppressor/cytotoxic phenotype (CD8) postgrafting is that the absolute number of CD4 cells is lower than normal in the first three to six months postgrafting, and the absolute number of CD8 cells is higher than normal in the first six months postgrafting resulting in lower than normal helper-inducer/suppressor-cytotoxic cell ratios.⁷⁸⁻⁸⁴ Similarly, the proportions of Fc-IgG receptor-bearing T cells (T_G, suppressor phenotype) were increased, and the proportions of Fc-IgM receptor-bearing T cells (T_M, helper phenotype) were decreased early after transplant

leading to decreased T_G/T_M ratios observed in recipients up to three years posttransplant.⁸⁵ Immature T cells or thymocytes (OKT10) were found circulating in small numbers up to six to 12 months postgrafting.^{78,80,81} The expression of HLA-DR (Ia-like) antigens was increased after three months postgrafting.⁸¹ Several groups have shown associations between viral infections and decreased CD4/CD8 ratios,^{81,84} but other groups have not shown such an association.^{79,80} In autologous marrow graft recipients it has been suggested that low CD4/CD8 ratios may be predictive of CMV infections.⁸⁶ Some groups showed a correlation between low CD4/CD8 ratios and acute GVHD.^{82,83} However, other studies indicate that most short-term autologous marrow recipients have low CD4/CD8 ratios.⁸⁷ Long-term recipients with chronic GVHD persist in having low proportions of CD4 cells and high proportions of CD8 cells.^{11,78,82,88}

The expression of IL 2 receptors, identified by the MoAb anti-TAC,^{89,90} on T cells from marrow recipients less than six months postgrafting is controversial. One group did not find IL 2 receptor positive T cells,⁸¹ whereas a more recent report showed >25% of the cells in the peripheral blood of marrow recipients undergoing graft rejection or acute GVHD were IL 2 receptor positive.⁹¹ In our studies peripheral blood lymphocytes from five of 12 short-term recipients with or without GVHD between 45 and 83 days postgrafting expressed IL 2 receptors ranging from 1.0% to 12.3% (Lum, unpublished). The expression of IL 2 receptors may represent in vivo activation of T cells by any number of clinical events or treatments. Investigators have found that OKT10 and Ia were expressed on the T cells from short-term recipients.^{78,80,81,84} Inverted CD4/CD8 ratios and the expression of OKT10 and Ia antigens may simply reflect immature and/or regenerating T cell populations early after marrow grafting with CD8 cells arising first. The effects of chronic GVHD on this sequence of events are discussed later.

In vivo cellular immunity as measured by delayed-type hypersensitivity is impaired in the first 100 days postgrafting.⁹² In vitro proliferative responses after stimulation with mitogens, specific antigens, and alloantigens measured by ³H-thymidine uptake are low during the first 100 days postgrafting.^{7-10,12}

A number of investigators have used a variety of in vitro systems for testing the ability of T and B cells from short-term marrow recipients to produce immunoglobulin after nonspecific activation with agents such as pokeweed mitogen or Protein A from *Staphylococcus aureus*.⁹³⁻⁹⁸ T cells from two thirds of the recipients did not provide T cell helper activity and had increased suppressor T cell activity.⁹⁵ More recent studies designed to identify T cell subsets responsible for abnormalities in T cell function show that CD4 cells failed to help and frequently exhibited suppression, and CD8 cells did not routinely exert suppressor activity.⁹⁹ Overall, T cells failed to help their B cell counterparts during the early period postgrafting to produce immunoglobulin. Such functional data indicate that T cells are not ready to perform in a concerted fashion with B cells early after grafting. In addition, T cells responsible for suppressor activity directed at Epstein-Barr virus (EBV)-driven B cell clones may not function, thereby permitting the development of B cell

lymphomas early after transplantation in those receiving T cell-depleted bone marrow. This notion is supported by observations in our laboratory showing that EBV-driven immunoglobulin secretion by donor B cells is often not suppressed by recipient T cells, whereas normal T cells do suppress the EBV-driven immunoglobulin secretion response of B cells.¹⁰⁰

T CELL-MEDIATED IMMUNITY IN LONG-TERM RECIPIENTS

After one year postgrafting, marrow recipients without chronic GVHD normalize their CD4/CD8 ratios, but those with chronic GVHD have persistently elevated proportions of CD8 cells with decreased proportions of CD4 cells in their peripheral blood.^{11,81,88} Low numbers of circulating CD4 cells in recipients with chronic GVHD may be a result of selective in vivo sequestration and/or depletion by ongoing GVHD reactions occurring in the skin, liver, or gut. This inverted ratio is associated with a number of in vitro findings described in the following sections.

Greater than 90% of marrow recipients make at least one positive response to a battery of five recall skin test antigens by four years postgrafting. Proliferative responses to mitogens, alloantigens, and specific antigens return to normal or near normal values after six months to one year postgrafting in recipients without chronic GVHD.⁹²

Early studies performed in the pokeweed mitogen system using T cells from long-term marrow recipients with and without chronic GVHD showed that roughly one half of those with chronic GVHD had T cells that failed to provide helper activity and exhibited excessive suppressor activity.^{88,101,102} Fractionation of T cells from recipients with chronic GVHD into Fc-IgG receptor positive T cells (T_H^+) and Fc-IgG receptor negative T cells (T_G^-) showed heterogeneous helper and suppressor activity in both T cell subsets.¹⁰³ Such T cell defects were less frequent in healthy long-term survivors. One study showed increased spontaneous Ig secretion by B cells from recipients with chronic GVHD.¹⁰²

Later studies showed that excessive suppressor T cell activity, for the most part, was due to increased proportions of CD8 cells. CD4 cells from one third of the patients with chronic GVHD did not provide helper activity,^{88,104} whereas CD4 cells from healthy long-term survivors consistently provided helper activity in the pokeweed mitogen system. Studies using immunoglobulin production assays show heterogeneous functional responses in the CD4 and CD8 T cell subsets from recipients with and without chronic GVHD.

In a recent study, tetanus toxoid, herpes simplex type 1 virus, and pokeweed mitogen were used to develop functional profiles of CD4 and CD8 subsets from long-term marrow graft recipients.¹⁰⁵ The study showed that immune reconstruction occurs in a variable fashion associated with extremely heterogeneous T and B cell subset functions. Recipients with chronic GVHD had delayed reacquisition of normal function within each T cell subset.¹⁰⁵ The spectrum of functional responses found in the T cell subsets may reflect developmental stages within each phenotypic lineage postgrafting. However, these nonspecifically stimulated functional assays of immunoglobulin production did not correlate

with *in vivo* specific antibody titers to tetanus toxoid or herpes simplex virus on a per patient basis.¹⁰⁵

B CELL-MEDIATED IMMUNITY EARLY POSTGRAFTING

Levels of IgG and IgM in the serum return to normal after three to four months postgrafting, but levels of IgA may remain low for years.^{7-10,12} IgE levels have been reported by several groups to be elevated in association with acute GVHD.¹⁰⁶⁻¹⁰⁸ Levels of the total hemolytic complement, the third component of complement, and the fourth component of complement are normal in the first three months postgrafting.^{12,28} Allogeneic and syngeneic marrow recipients have severely depressed antibody responses to immunizations with the neoantigens keyhole-limpet hemocyanin, pneumococcal polysaccharide type 3 antigen, and bacteriophage OX174 in the first six months postgrafting.^{12,13,15} An early study used specific antibody titers to keyhole-limpet hemocyanin and yellow fever virus as immunologic probes in a short-term marrow recipient to prove lymphoid engraftment after transplantation from an identical twin donor who was immunized to keyhole-limpet hemocyanin and yellow fever virus pretransplant.¹⁰⁹ The recipient developed antibody titers to keyhole-limpet hemocyanin. Recent data from this and other laboratories show that IgG antibody titers to recall antigens such as tetanus toxoid, diphtheria toxoid, and measles virus can be detected within the first 100 days postgrafting with or without booster immunizations to the test antigens.¹¹⁰⁻¹¹² Two hundred and twenty-one of 235 (94%) short-term patients had titers to tetanus toxoid, 176 of 232 (76%) short-term recipients had titers to diphtheria toxoid, and seven of eight (87%) short-term recipients had titers to measles virus¹¹² (Lum, unpublished). Specific antibody titers to recall antigens in short-term marrow recipients were in the normal range without booster immunizations, whereas responses to primary and secondary immunizations with neoantigens postgrafting were severely depressed during the early period postgrafting.

The source of the specific antibodies to recall antigens during the first 100 days remains unclear. The likely source of antibody is B cells of donor origin. However, persistent host antibodies, persistent host B cells producing antibody, passively acquired antibody via blood product support, and the stimulation of already precommitted memory B cells transferred from the donor may all contribute to antibody titers to recall antigens. Antigen-specific helper activity may not be required for *in vivo* antibody synthesis by precommitted B cells. This hypothesis is supported by one report that shows that titers to tetanus toxoid and hepatitis B are detectable in short-term recipients who received T cell-depleted marrows by immunizing donors alone or both the donors and the recipients pretransplant.¹¹³

The probability of detecting host-type isohemagglutinin in ABO-incompatible marrow graft recipients is less than 0.20 by day 80 postgrafting and is zero by day 120 postgrafting.¹³ Therefore, the levels of specific antibody to recall antigens detected at 100 days postgrafting suggest a major contribution produced by transferred donor B cells. Immunoglobulin allotypes of donor origin have been detected as early as 113 days postgrafting.¹¹⁴ Clearly, the synthesis of antibodies of

donor allotype must begin before this time to be detected without interference from host-type allotypes.

B cell phenotyping data in the first two months postgrafting show that B cells from allogeneic marrow recipients have normal or near normal numbers of surface immunoglobulin positive (sIg⁺) B cells as measured by polyvalent antisera directed at human immunoglobulin.^{9,10,12} However, recent phenotyping data shows the appearance of B cells bearing the CD5 T cell antigen 30 days postgrafting.¹¹⁵ The CD5⁺ B cells were present in healthy recipients. High proportions of CD5⁺ B cells are found in fetal spleen (30% to 50%).⁹¹ These B cells may represent an early stage of differentiation of normal B cells or a separate lineage.¹¹⁶ Data from this laboratory show that populations of sIgG, sIgM, sIgA, or sIgD⁺ B cells in peripheral blood lymphocytes may be absent in certain recipients (Lum, unpublished). The most likely explanation for these findings is that B cells lacking a specific surface marker may be associated with specific maturational defects. This notion is supported by a study that showed B cells from marrow recipients had higher numbers of primitive μ -determinants per B cell than normal B cells¹¹⁷; these findings are similar to those found in murine splenic B_H cells that display increased μ -determinants early in ontogeny.

B cell-enriched populations from marrow recipients in the first three months postgrafting fail to produce *in vitro* antibody after stimulation with specific or nonspecific activators in the presence of normal T cells.^{93-95,97,118} B cells from a few marrow recipients in the early period produced nonspecific immunoglobulin after EBV (T-independent) stimulation.⁹⁷ Stimulation with EBV to polyclonally activate production of IgG antitetanus toxoid antibodies (anti-TT) was not successful during the early period.¹¹⁹ Furthermore, B cells from short-term recipients failed to produce anti-TT after stimulation with tetanus toxoid in the presence of donor helper T cells.¹¹⁸

In assays for B cell activation, proliferation, and differentiation, B cells from short-term marrow recipients cannot be activated by staphylococcal aureus Cowan I bacteria (SAC) to subsequently proliferate or differentiate in the presence of normal T cell factors containing B cell growth factor (BCGF), B cell differentiation factor (BCDF), and IL 2.¹²⁰ With the exception of preprogrammed spontaneous antibody secretion, the B cells from marrow recipients seem incapable of responding to exogenous signals.

B CELL-MEDIATED IMMUNITY IN LONG-TERM RECIPIENTS

Primary and secondary responses to neoantigens (keyhole-limpet hemocyanin and bacteriophage OX174) recover to normal levels by one year in healthy recipients but remain impaired in those with chronic GVHD. Those with chronic GVHD not only have defective primary responses to bacteriophage OX174, but they fail to switch from IgM production to IgG production in secondary responses.¹³

Recent studies using recall antigens have been informative. In healthy long-term recipients, 85 of 125 (68%) studied had IgG titers to tetanus toxoid; 62 of 104 (60%) had IgG titers to diphtheria toxoid, and 12 of 18 (66%) had IgG titers to measles virus. In recipients with chronic GVHD, 79 of 166

(48%) studied had IgG antibody titers to tetanus toxoid; 40 of 136 (29%) had IgG titers to diphtheria toxoid, and 6 of 15 (40%) had IgG titers to measles virus¹¹² (Lum, unpublished). None of the recipients (ranging from one year to nine years postgrafting) were boosted to the test antigens postgrafting. Furthermore, a number of long-term recipients who received T cell-depleted marrow grafts also had detectable antibody titers to tetanus toxoid and diphtheria toxoid.¹²¹ Such data show that there was transfer of antigen-specific B cell memory from the marrow donors to the marrow recipients, and the transferred immunity persisted for years posttransplant. One report showed transfer of anti-acetylcholin-receptor immunity from a marrow donor with myasthenia gravis to the marrow recipient who developed myasthenia gravis postgrafting.¹²² Recent studies show that bone marrow mononuclear cells could produce specific antitetanus toxoid antibody after in vitro stimulation with pokeweed mitogen or tetanus toxoid with or without tetanus toxoid booster immunizations.^{123,124}

B cells from three fourths of the recipients with chronic GVHD have impaired B cell function, whereas only one third of the healthy recipients have B cell defects in in vitro immunoglobulin production systems stimulated with pokeweed mitogen.^{81,82,85,94,97,101} Furthermore, B cells from the same marrow recipient have different responses to different activators of immunoglobulin production when studied at the same time in systems activated by pokeweed mitogen, EBV, tetanus toxoid, and herpes simplex type 1 virus.¹⁰⁵ These suggest that there are functionally distinct groups for each activator of polyclonal immunoglobulin production that mature at different times postgrafting. Although the above studies document B cell failure, none specify the types of defects responsible for the lack of immunoglobulin secretion. These results provided the rationale in our laboratory for developing assays for assessing antigen-specific immunity and identifying the types of B cell defects that occur in marrow recipients.

Our latest studies focused on antigen-stimulated specific antibody synthesis in lymphocytes obtained from long-term recipients. Anti-TT synthesis was induced using a tetanus toxoid-specific system.¹²⁵ An early study showed that tetanus toxoid-specific T cell helper activity can be detected in cocultures of recipient T cells and donor B cells.¹¹⁸ However, recipient B cells cocultured with donor T cells failed to produce anti-TT. The results of this study suggest that antigen-specific T cells can be transferred in marrow transplantation. In another study designed to rule out the possibility of low antigen-specific B cell precursor frequency, we used high numbers of T and B lymphocytes per culture and found that only a minority of marrow recipients had B cells capable of producing in vitro anti-TT when increased numbers of lymphocytes were placed in culture.¹²⁶ These studies demonstrated that antigen-specific precursor frequency is low in long-term marrow recipients. Roughly one third of long-term marrow recipients had B cells capable of producing anti-TT after EBV stimulation.¹¹⁹ These data suggested that the majority of B cell defects were primarily due to deficient function rather than a low antigen-specific B cell precursor frequency.

In assays that assess nonspecific B cell activation, proliferation, and differentiation, B cells from long-term marrow recipients exhibited two patterns in their B cell functions.¹²⁷⁻¹²⁹ B cells from some recipients could be activated with SAC but had decreased proliferative and differentiative responses to T cell supernatants containing B cell growth and differentiation factors. B cells from other recipients could proliferate but could not synthesize immunoglobulin after SAC activation and stimulation with T cell supernatants. Healthy long-term recipients had fewer, less severe B cell defects in these B cell function assays. Such B cell defects were not observed in the normal control subjects. There was a high degree of correlation ($P = 0.0075$) between low IgG levels in marrow recipients and the failure of B cells from marrow recipients to synthesize IgG after costimulation with SAC and T cell supernatants.¹²⁰ These studies suggest that the normal maturation of B cells is interrupted by specific blockades at one or more stages after marrow grafting.

ATTEMPTS TO ACCELERATE RECONSTITUTION

Attempts to accelerate the immune reconstitution with the use of thymic tissue transplantation¹³⁰ or thymosin fraction V¹³¹ have not been successful nor have they influenced the development of chronic GVHD thought to be due to low or absent thymic tissue function in older (>30 years) marrow graft recipients. However, the results of such studies indicate that future administration of thymic hormones or related molecules may be done safely. Other hormones that might be considered for use in immune reconstitution are thymosin alpha-1,¹³² the pentapeptide thymopoietin,¹³³ and serum thymic factor.¹³⁴ Varicella zoster-specific transfer factor did not reconstitute varicella zoster-specific immunity or prevent recurrent varicella zoster infections when given between day 50 and day 64 postgrafting.¹³⁵

The development and availability of recombinant T and B cell growth factors will permit clinicians to administer specific molecules to determine if certain T and B cell immune defects could be constituted in marrow recipients and other patients with immune deficiency disorders.

REIMMUNIZATIONS FOR MARROW RECIPIENTS?

Most transplant centers have not reimmunized patients postgrafting. However, questions that are frequently asked include: (1) Do transplant recipients need to be routinely reimmunized to the childhood series of immunizations? (2) Should they be immunized to specific pathogens such as *Streptococcus pneumoniae* or *Hemophilus influenzae*? (3) Should live viral vaccines be used in the reimmunization of marrow recipients?

Recent studies that show transfer of antigen-specific immunity from the marrow donors to marrow recipients suggest that a significant number of marrow recipients will develop specific antibody titers to recall antigens without reimmunization to the specific antigens. Obviously, those who develop antibody titers need not be reimmunized to the specific antigens. However, those who do not develop antibody titers need to be considered for reimmunizations with diphtheria toxoid, tetanus toxoid, and inactivated polio vac-

cines and followed for the development of specific antibody titers. Herd immunity and transferred specific immunity may help explain why more marrow recipients do not succumb to measles, polio, tetanus, diphtheria, and pertussis infections. Vaccinations with live viral vaccines are not recommended. The inactivated Salk polio vaccine would be useful for those at risk during outbreaks of polio. The value of specific viral or bacterial vaccines (herpes vaccine, Pneumovax) in marrow recipients remains debatable. In some immunodeficiency states the efficacy of such immunizations is poor.

Furthermore, it is unclear how many immunizations with a specific vaccine would be necessary to obtain specific immune responses in the transplant population. Those undergoing reimmunization schedules would likely require two or three immunizations before they would develop circulating antibody titers. Recipients being treated for active chronic GVHD should have their immunizations deferred until their disease is resolved, since it is likely that they would not respond to immunizations. Titers to the specific antigen should be followed to determine if an immune response was obtained.

RECAPITULATION OF ONTOGENY

Immune reconstitution after human marrow grafting has offered a unique model for studying stages of normal immune ontogeny, transfer of immunity, and immune defects that may parallel those seen in immunodeficiency disorders. The kinetics of immune reconstitution follow a general pattern. First to appear are cytotoxic and phagocytic cells. More mature systems characterized by highly specialized cellular functions such as proliferation, helper activity, differentiation, lymphokine production, and antibody biosynthesis appear later. The latter functions require complex and finely tuned cell-cell interactions mediated by cell contact and soluble factors. As the elements of the immune system regain their roles, healthy long-term recipients develop immunocompetence sooner than those with chronic GVHD. During the recapitulation of ontogeny over a time period ranging up to four years postgrafting, a spectrum of functional and nonfunctional stages within various T and B cell phenotypes are expressed. The development of immunocompetence includes antigen-specific immunity transferred from the marrow donor to the marrow recipient as shown by (1) the detection of specific antibody titers to recall antigens and (2) the ability of T and B cells from long-term recipients

to produce specific recall antibodies *in vitro*. The overall pattern of immune reconstitution is delayed in recipients with chronic GVHD. The delay may be due to failure of lymphocyte subsets to progress through certain developmental stages. On the other hand, the syndrome of chronic GVHD with autoantibody formation and other autoimmune phenomena may be a result of immune dysregulation due to unregulated overactivity of certain lymphocyte subsets. (Perhaps with appropriate thymic education this would not occur.)

For the sake of discussion, the immune reconstruction process was artificially divided into early and late phases and into the various cell types. However, one must emphasize that synchronization of various components of the lymphoid system is essential for complete reconstitution. Without appropriate synchronization of the various elements of the immune system the result would be immune dysfunction. Of the multiple factors studied for their effects on immune reconstitution, time continues to be a key factor affecting the reestablishment of immunocompetence. It remains unclear whether certain immune deficits lead to the development of GVHD or immune deficits are a result of GVHD or its treatment. Furthermore, the effects of T cell depletion of bone marrow or HLA-mismatched transplantation on immune reconstitution are unknown and remain areas of active investigation.

FUTURE DIRECTIONS

The questions that emerge from this review suggest future laboratory and clinical investigations revolving around the following issues: (1) augmenting specific immunity to pathogens before and/or after transplant by administering vaccines or vaccine substitutes, (2) the clinical use of growth factors to restore specific T and B cell functions, (3) the use of adoptively transferred donor-derived cytotoxic T cell lines directed at specific viral or bacterial pathogens to treat recipients with specific infections, and (4) the insertion of specific genes into the bone marrow inoculum using retroviral vectors to reconstitute specific immune defects. These maneuvers are approaches that may accelerate immune reconstitution and shorten the immunodeficiency experienced by marrow recipients.

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Plasminogen Activator Inhibitors

By E.D. Sprengers and C. Kluft

PROTEOLYTIC DEGRADATION of fibrin clots (fibrinolysis) is mediated by the enzyme plasmin. Plasmin is formed in the circulation from the inactive precursor plasminogen through the proteolytic action of plasminogen activators (PA).^{*} For both plasmin and plasminogen activators specific inhibitors are present: α_2 -antiplasmin and plasminogen activator inhibitors (PA-inhibitors), respectively (Fig 1).

Proteinase inhibitors represent nearly 10% of the total protein in blood plasma.¹ They control a variety of critical events associated with connective tissue turnover, coagulation, fibrinolysis, complement activation, and inflammatory reactions. As a general rule, proteinase inhibitors can react with several proteinases. The attribution of a specific function to a certain proteinase inhibitor depends on its concentration and its second-order rate constant with a certain proteinase; these two parameters determine the half-life of a proteinase due to inactivation by the inhibitor. As early as 1963 Brakman and Astrup claimed the existence of PA-inhibitors by demonstrating a fibrinolytic inhibitor that did not inhibit plasmin in the blood of pregnant women.² Several reports about the presence of a specific inhibitor of PA in human plasma have appeared before 1982; however, due to the lack of appropriate kinetic data, a specific inhibitor of PA in blood plasma has never been convincingly demonstrated.^{3,4} The question has been raised whether there is a logical function for specific PA-inhibitors at all, since the activity of PA is regulated by several mechanisms such as release from the vessel wall and rapid hepatic clearance, specific interaction with fibrin (at least for tissue-type PA [t-PA]) and proteolytic activation of single-chain PA (at least for pro-urokinase). Moreover, the activity of the fibrinolytic system is strongly regulated by the abundant plasmin inhibitor α_2 -antiplasmin (0.06 mg/mL plasma). Nevertheless, the existence of specific PA-inhibitors is now no longer a matter of dispute. PA-inhibitors can already be classified in at least three immunologically different groups⁵: the endothelial cell type PA-inhibitor (PAI-1), the placental type PA-inhibitor (PAI-2), and protease nexin-I.^{*} Their function in the regulation of the activity of the fibrinolytic system is still only partly understood. This review is not meant as a historical or chronological review, but briefly summarizes what is presently known about PA-inhibitors.

TYPE 1 PA-INHIBITOR

Endothelial cell-type PA-inhibitor (PAI-1) is synthesized by endothelial cells⁶⁻¹³ and human hepatocytes in culture,¹⁴ by several hepatoma cell lines,¹⁴⁻¹⁶ granulosa cells,¹⁷ SV-40 virus-transformed human lung fibroblasts (the cell line WI-38 VA 132 RA),¹⁸ the MJZJ melanoma cell line,¹⁹ and probably by vascular smooth muscle cells²⁰; it is also found in the α -granules of blood platelets^{18,21-23} and plasma.²⁴⁻⁴⁰ This inhibitor reacts with both urinary-type plasminogen activator (u-PA) and t-PA, with a second-order rate constant of above 10^6 to 10^7 mol/L⁻¹ s⁻¹.^{24,25,27,28,30,40} The reaction of the inhibitor with these PA eventually results in the formation of sodium dodecyl sulphate (SDS)-stable complexes with a molecular mass that is 40 to 50 kilodaltons (kd) higher than the molecular mass of the free PA.^{10-14,20,22,24,27,28,31-34,36,38,40}

The PA/PA-inhibitor complex has no PA activity as such, but after SDS/Triton X-100 treatment a PA activity can be generated that is much smaller than the activity of an equivalent quantity of free PA.^{10-12,24,26-28,32-34,40,41} Whether this is due to dissociation of a small part of the PA/PA-inhibitor complex molecules induced by the SDS/Triton X-100 treatment or due to an intrinsic activity of the complex after SDS/Triton X-100 treatment is not yet clear.

Endothelial cell-conditioned medium and blood platelets contain both active and inactive (latent) forms of PAI-1.^{12,23,38,42,43} The PA-inhibitor is probably synthesized by cultured endothelial cells in an active form. Inactivation (apart from complex formation with PA) can occur in at least two different ways: by a rapid inactivation of newly synthesized PA-inhibitor upon leaving the cell, with a calculated half-life of 0.29 hour; and by a much slower inactivation in (cell-free) conditioned medium with a half-life of two to four hours at 37 °C.⁴⁴ Whether the rapid and slow inactivation processes also take place in vivo is not yet clear. The accumulation of the inactive (latent) form in 24-hour endothelial cell-conditioned medium, reaching up to a hundredfold excess of inactive over active PA-inhibitor,⁴⁵ is probably due to inactivation of actively synthesized PA-inhibitor.⁴⁴ The inactive (latent) form does not inhibit t-PA or u-PA, nor does it form complexes with these PA. This inactive form can, however, be "activated" in vitro by the action of protein denaturants such as SDS, urea, and guanidine HCl, followed by renaturation through removal of the

^{*}At their meeting of June 2, 1986, in Jerusalem, Israel, the Subcommittee on Fibrinolysis of the ICTH proposed the designation of PA-inhibitors by sequence numbers. Endothelial cell-type PA-inhibitor (nomenclature of 1985⁵) was proposed to be designated as plasminogen activator inhibitor 1 (PAI-1), and placental-type PA-inhibitor (nomenclature of 1985⁵) was proposed to be designated as plasminogen activator inhibitor 2 (PAI-2). Protease nexin I maintains its name. In this review we use the newly proposed nomenclature.

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PROTEASE NEXIN

Protease nexin I has been purified to homogeneity from human foreskin fibroblast-conditioned medium.⁶⁰⁻⁶² It has a molecular mass of about 43 kd and contains approximately 6% carbohydrate. It is a broad specificity inhibitor of trypsin-like serine proteinases. It reacts rapidly with trypsin ($k_{on} = 4.2 \times 10^6 \text{ mol/L}^{-1}\text{s}^{-1}$), thrombin ($k_{on} = 6.0 \times 10^5 \text{ mol/L}^{-1}\text{s}^{-1}$), u-PA ($k_{on} = 1.5 \times 10^5 \text{ mol/L}^{-1}\text{s}^{-1}$), single-chain t-PA ($k_{on} = 1.5 \times 10^3 \text{ mol/L}^{-1}\text{s}^{-1}$), two-chain t-PA ($k_{on} = 3.0 \times 10^4 \text{ mol/L}^{-1}\text{s}^{-1}$), and plasmin ($k_{on} = 1.3 \times 10^5 \text{ mol/L}^{-1}\text{s}^{-1}$), forming 1:1 complexes with these proteinases.⁶¹ It contains a high-affinity heparin binding site; in the presence of heparin the rate of the reaction of protease nexin I with thrombin is nearly diffusion-controlled.⁶¹

Protease nexin I is synthesized by a number of anchorage-dependent cells including fibroblasts, heart muscle cells, and kidney epithelial cells.⁶³ In serum-free cultures of human fibroblasts, secreted protease nexin I suppresses the cellular sensitivity to the mitogenic action of added thrombin,^{64,65} limits activation of secreted single-chain u-PA by exogenous proteinases^{66,67} and inhibits two-chain u-PA.^{60,66}

Fibroblasts bear surface receptors that mediate the endocytosis and lysosomal degradation of proteinase/protease nexin I complexes.⁶⁷ Protease nexin I is not detectable in blood plasma. Since the second-order rate constants of protease nexin I with thrombin and trypsin are much higher than with either t-PA or urokinase, protease nexin I cannot be considered a true PA-inhibitor. Also, because data on protease nexin I concentrations are lacking, a calculation of a half-life of plasminogen activators due to inactivation by protease nexin I cannot be made.

PHYSIOLOGY OF PA-INHIBITORS

PA play a central role in a number of physiologic processes. Of these, the fibrinolytic system has received the most attention. For this reason, most literature on PA-inhibitors deals with the role of these inhibitors in the homeostasis of the circulation. Very little is known about the function of PA-inhibitors in the many other physiologic processes in which PA are involved (tissue repair, nidation, fertilization, cell proliferation, etc).

In most studies, only PA-inhibitory activity has been measured in assays based on titration of PA-inhibitors with PA. The reader should be cautioned that the interpretation of these assays is not always easy, since increased PA-inhibitory activity can be caused by either increased PA-inhibitor or decreased PA. Also in most studies immunologic data on the type of PA-inhibitor are lacking. For this reason we use in this review the vague term "PA-inhibitory activity" when more precise data are lacking. Otherwise the term will be more specified.

In pooled normal human blood plasma PAI-2 and protease nexin I are not found. About 60% of the PA-inhibitory activity is PAI-1. The remaining 40% is attributed to a so far limitedly characterized factor, designated as PA-binding protein.^{39,68}

The PA-inhibitory activity in blood plasma of healthy individuals is subject to a diurnal fluctuation; lowest values

are found at 3:00 PM.⁶⁹ Moreover, there is a large interindividual variation of plasma PA-inhibitory activity levels (at 9:00 AM) ranging from 0.0 to 1.3 nmol/L.^{18,25,27-30,39} On the other hand, the 9:00 AM value of plasma PA-inhibitory activity of a certain individual is constant for at least several weeks (Sprengers et al, unpublished results, January 1986). As mentioned before, the cellular origin(s) of the plasma PA-inhibitor has not yet been established.

In vitro, PAI-1 is not stable at 37°C. Incubation of endothelial cell-conditioned medium, partly purified inhibitor, or plasma at this temperature leads to a first-order decrease of PA-inhibitory activity with a half-life of two to four hours.^{23,68,70} Activated protein C and thrombin inactivate the PA-inhibitory activity in endothelial cell-conditioned medium, an effect that is at least partly due to a direct effect of activated protein C (and thrombin) on the inhibitor.⁷⁰⁻⁷³ Activated protein C can form 1:1 complexes with PA-inhibitor.⁷² This process may contribute to another linkage of the coagulation and the fibrinolytic system. The antithrombotic effect of activated protein C mediated by inhibiting the coagulation pathway may thus be enforced by its profibrinolytic potential.

PA-inhibitory activity is very rapidly cleared from the circulation; half-lives of seven minutes in the rabbit⁷⁴ and 3.5 minutes in the rat have been reported.⁷⁵ The inhibitory activity is cleared at a much slower rate during liver ligation, suggesting that clearance takes place primarily in the liver, as is true also for PA.⁷⁵ It is interesting to note that though the steady state level of PA-inhibitor is several orders of magnitude lower than that of the major plasma proteinase inhibitors, this short half-life suggests that the rate of synthesis of PA-inhibitor is approximately of the same order of magnitude as of the major plasma proteinase inhibitors.

PAI-1 in blood plasma behaves as an acute-phase reactant protein.^{76,77} PA-inhibitory activity rapidly increases after major surgery, myocardial infarction, and severe trauma. We observed that PA-inhibitory activity was only increased on the first postoperative day, thus being the most rapidly reverting acute-phase reactant protein described so far.⁷⁷ Other authors observed a much more slowly reverting pattern, however.⁷⁶ According to the latter authors, the acute-phase response parallels the response of cortisol after extensive surgery.⁷⁸ The administration of adrenocorticotropin (ACTH), however, induces cortisol to the same extent while leaving PA-inhibitory activity unaffected. This excludes a simple cause-effect relationship between increased cortisol and PA-inhibitory activity levels.⁷⁸

PA-inhibitory activity in plasma is also induced by infusion of a single dose of bacterial wall lipopolysaccharide (endotoxin) or interleukin-1.^{74,79} The increase is optimal in about four hours and is reverted within 24 hours. Lipopolysaccharide and interleukin-1 added to endothelial cell culture yield a tremendous increase in PA-inhibitory activity in the conditioned medium of the endothelial cells.^{74,79,80} It is significant that interleukin-1 does not increase PA-inhibitory activity in the conditioned medium of hepatocytes and the human hepatoma cell line Hep G2, suggesting endothelial cells rather than liver as the source of increase in PA-

inhibitory activity in plasma by the acute-phase reaction mediator interleukin-1.⁷⁹ Thrombin added to confluent monolayers of human endothelial cells resulted in a sixfold increase in PA-inhibitory activity in 24-hour-conditioned medium, an effect that was dependent on RNA and protein synthesis.⁸¹ Although PA-inhibitor antigen was not assessed in this study, the authors suggest that because t-PA antigen was also increased, the increase in PA-inhibitory activity was due to an increased amount of PA-inhibitor antigen. Since thrombin inactivates PA-inhibitory activity in endothelial cell-conditioned medium⁷⁰ this observation can only be explained by assuming that the increased rate of synthesis of PA-inhibitor is higher than the increased rate of inactivation. Indeed, we recently demonstrated that low concentrations of thrombin added to endothelial cell cultures increase both PAI-1 antigen and activity in the conditioned medium, whereas high concentrations of thrombin increase PAI-1 antigen but decrease its activity in the conditioned medium due to proteolytic degradation of the inhibitor (Van Hinsberg et al, unpublished data, September 1986).

Long-term oral administration of the synthetic anabolic steroid stanozolol to human volunteers decreases the PA-inhibitory activity in blood plasma.⁸² At least 3 weeks are required for maximal suppression of PA-inhibitory activity using a dosage of 10 mg/day.⁸² Treatment with stanozolol, 10 mg orally per day for 2 weeks preoperative, significantly enhanced the fibrinolytic activity before surgery but did not prevent the decline in fibrinolytic activity which occurs after surgery.⁸³ In cell culture the synthetic corticosteroid dexamethasone induces PA-inhibitor in HTC rat hepatoma cells.^{15,16} However, in the human hepatoma cell line Hep G2 dexamethasone does not influence the secretion of PAI-1.¹⁴

During pregnancy, PA-inhibitory activity gradually increases up to tenfold at the end of the third trimester.⁸⁴⁻⁸⁶ Postpartum it rapidly returns to prepregnant values. In "at risk" pregnancies (preeclampsia, suspected fetal growth retardation, diabetes mellitus, thrombosis and/or a previous history of thrombosis) a considerably wider range of PA-inhibitory activities, including much higher values, is observed.⁸⁴ Using an immunologic assay, a gradual increase in PAI-2 has been reported during pregnancy, yielding values up to 100 $\mu\text{g}/\text{mL}$ in the third trimester and rapidly returning to nonpregnant values after delivery.⁵⁹ Moreover, by immunoadsorption using a monoclonal anti-PAI-2 antibody, PA-inhibitory activity can be isolated from pregnancy plasmas, suggesting that PAI-2 in pregnancy plasmas is at least partly active.⁵⁹ Unfortunately, in this study no data on the molecular forms or specific activity (units/mg antigen) of the PA-inhibitor are given; so a precise interpretation of the data, ie, whether all the antigen represents active PA-inhibitor, is not possible. Our results indicate that the increase in PA-inhibitory activity during pregnancy as assessed by a titration assay can be almost fully quenched by a specific anti-PAI-1 antibody (Sprengers et al, unpublished data, April 1985).

Several authors studied associations of PA-inhibitory activity levels with certain states of disease. The most intensively studied pathologic state so far is deep venous thrombosis. Three groups independently established that

deep venous thrombosis is frequently (but not necessarily) associated with high PA-inhibitor levels.⁸⁷⁻⁹⁰ Paramo et al recently found that preoperative levels of PA-inhibitory activity were significantly higher in patients that developed postoperative deep venous thrombosis.⁸⁹ On the other hand, deep venous thrombosis is also frequently associated with an impaired release of t-PA from the vessel wall.^{87,88} These data indicate that impaired fibrinolysis can play a role in the pathogenesis of deep venous thrombosis. In patients with myocardial infarction several investigators have found an increased PA-inhibitory activity together with a decreased level of t-PA activity or antigen.^{29,91-94} In patients with coronary artery disease, the increase in the level of the PA-inhibitory activity did not correlate with the severity of the disease, however.⁹⁵ High PA-inhibitory activity levels are positively correlated with hyperlipoproteinemia.⁹⁶ Other authors found a correlation between high PA-inhibitory activity and levels of serum triglycerides.⁹³ Juhan-Vague et al found increased PA-inhibitory activity levels in the plasma of critically ill patients with a variety of diseases: pancreatitis (four of eight), liver disease (three of eight), malignancy (five of 26), myocardial infarction (five of 28), and deep venous thrombosis (two of nine).²⁹ Kruithof et al also reported an increased PA-inhibitory activity in an even wider variety of hospitalized subjects.⁹² To the contrary, in terminal renal insufficiency, either in patients with nonfunctioning kidneys *in situ* or without kidneys, PA-inhibitory activity was not significantly different from normal values.⁹⁷

The general picture emerging is that increased PA-inhibitor levels in blood plasma is indicative of acute illness as such, and that it is not associated with a special type of disease. In no case has a cause-effect relationship between increased PA-inhibitor levels and a certain disease been convincingly demonstrated, although at least in the case of deep venous thrombosis PA-inhibitory activity might have some prognostic value.⁸⁹

In normal human blood at least two pools of PA-inhibitory activity are present.²³ One pool is the (platelet-poor) plasma pool that shows a very rapid turnover and for which the normal values show a very large interindividual variation ranging from 0.0 to 1.3 nmol/L. The other pool is the platelet PA-inhibitor pool that probably shows a turnover of the order of magnitude of the turnover of platelets themselves (half-life three to four days) and for which the normal values show a much smaller range (mean: 6.7×10^{-14} mole/platelet; SD = 3.0×10^{-14}).²³ It can be calculated that in a given volume of blood the PA-inhibitory activity in the platelets is about four times higher than in the plasma.²³ The platelet PA-inhibitory activity is probably present in the α -granules^{21,22} and is indistinguishable from PAI-1 in endothelial cells.²³

During coagulation, the PA-inhibitor derived from platelets may play a very important role in protecting the blood clot against premature lysis. The high concentration of activated platelets in a blood clot may yield a local PA-inhibitor concentration that is several orders of magnitude higher than the value in plasma and would prevent too-sudden lysis. Instability of the PA-inhibitor at 37 °C (*in vitro* half-life about two hours) and inactivation by activated

protein C and thrombin may result in a gradual disappearance of the inhibitor from the clot. Subsequent diffusion of PA into the clot may result in clot lysis. Plasma PA-inhibitory activity might serve in systemic PA-activity control, preventing the unwanted action of PA under resting conditions.

The role PA-inhibitors play in thrombolytic therapy with PA remains to be established. A simple calculation shows that in order to maintain a steady-state concentration of PA-inhibitor of 1.0 nmol/L with a hypothetical half-life of ten minutes in man, a rate of synthesis of 0.45 mg/hr is required. This is far less than the amount of t-PA (up to 60

mg/hr) that is administered by intravenous injection as thrombolytic therapy to patients with myocardial infarction.^{98,99} This amount of PA-inhibitor as such is probably not a major factor in the success of thrombolytic therapy. On the other hand, since data on PA-inhibitors during thrombolytic therapy are presently lacking, conclusions cannot be drawn.

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Successful Graft-Versus-Host Disease Prevention Without Graft Failure in 32 HLA-Identical Allogeneic Bone Marrow Transplantations With Marrow Depleted of T Cells by Monoclonal Antibodies and Complement

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Thirty-two patients with acute leukemia, chronic granulocytic leukemia, or multiple myeloma received a T lymphocyte-depleted HLA-identical marrow. After being treated with pan-T monoclonal antibodies (MoAbs) and one round of baby rabbit complement, the mean percentage of T cell depletion was $94\% \pm 4\%$. The number of residual viable T cell infused to the patient was $0.99 \pm 0.65 \times 10^6$ per kg body weight. The patients were conditioned with fractionated total body irradiation (TBI) (12 Gy) preceding high doses of cyclophosphamide (120 mg/kg). Methotrexate was used as an additional immunosuppressant in the first ten patients. For the following 22 patients no posttransplant immunoprophylaxis was administered. Eight patients died within three months due to complications related to

transplantation. Engraftment was achieved in all evaluable patients, and no patient has a late graft failure. The proof of total chimerism was established in 24 patients. Twenty-four of 27 evaluable patients (88%) did not have an acute graft-versus-host disease (GVHD) greater than grade 0 to 1. Two patients had a grade 2 (skin only), and one patient had a grade 4 acute GVHD (the latter had only 80% of T cell depletion). A medullary relapse occurred in 11 patients (nine of them had previously been defined as "high risk leukemia"). Our data suggest that it may not be necessary to deplete nearly all T cells to prevent acute GVHD in recipients of HLA-identical marrow.

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ACUTE GRAFT-V-HOST DISEASE (GVHD) remains a major cause of morbidity and mortality in allogeneic bone marrow transplantation (BMT). Complex relationships that are not always well understood exist between GVHD and infectious complications posttransplant. Despite posttransplant immunosuppression, approximately 40% to 60% of patients who receive HLA-matched allogeneic marrow grafts develop acute GVHD. Among 1,106 patients analysed as part of the European Bone Marrow Transplant Group (EBMT), the acute GVHD grading distribution was as follows: mild in 23%, moderate in 20%, severe in 21%, and no GVHD in 36%. Chronic GVHD was observed in 24% of the patients (it was extensive in one third of these cases).¹

Murine studies have demonstrated that the removal of T cells from donor marrow can effectively prevent acute GVHD.² In man, OKT3 has not significantly reduced the incidence of acute GVHD in the first attempt to use opsonization of the T lymphocytes from the graft with monoclonal antibodies (MoAbs).^{3,4}

It has been generally considered that a near complete depletion of T cells from the marrow graft was necessary to decrease effectively the incidence of acute GVHD. Thus a large number of different methods have been reported with the aim of removing 99% of T cells from the marrow.⁵⁻⁹ Among these methods, the use of murine MoAbs specific for T lymphocyte antigens followed by complement-mediated

cytolysis is well adapted to clinical use. As a general rule, transplants following cyclophosphamide and total body irradiation (TBI) are not rejected from HLA-matched siblings, whereas the same is not true after an in vitro depletion of T cells prior to a bone marrow transplantation. According to several papers that have been published recently,^{7,10} an unusual percentage of graft failures occurs in recipients of a T cell-depleted marrow graft.

The results of an open pilot study on 32 patients transplanted with a HLA-identical bone marrow in vitro treated with pan-T MoAbs and one round of baby rabbit complement are reported here. Ten patients, included in a previously reported pilot study,¹¹ were treated with methotrexate plus T cell depletion. Acute GVHD was prevented by T cell depletion alone in 22 patients. Our aims were to evaluate the efficacy of incomplete T cell depletion as a method of preventing acute and chronic GVHD and evaluating the reconstituting ability of the T cell-depleted marrow. Our T cell depletion protocol was sufficient in preventing GVHD in most cases, and an engraftment occurred in all the study patients.

MATERIALS AND METHODS

Monoclonal antibodies and complement. According to their availability, we used different pan-T MoAbs with the intention of evaluating, in a nonrandomized study, two pan-T antibody combinations of different origins (both include a CD2 antibody). The first cocktail (OKT-pool) consisted of two murine pan-T antibodies OKT11 (CD2) and OKT3 (CD3) from Ortho (Raritan, NJ). Both MoAbs used were of IgG2 class and they were reactive with almost all mature T lymphocytes.¹² The second cocktail (CD-pool) was composed of two IgG2 class (CD5/A50 and CD7/121) and one IgM class (CD2/D66) MoAbs (A. Bernard, Institut Gustave Roussy, Villejuif, France).¹³ Neonatal rabbit complement (23 to 28 days) was prepared in our Institution (Immunological Department, Blood Transfusion Center, Besançon, France). The characteristics of the rabbit complement are detailed in Table 1.

Detection of residual T cells. Identification of the T cells coated by pan-T MoAbs was performed with indirect immunofluorescence; cells were counted either by microscope or flow cytometry. Cell viability was evaluated with trypan blue or ethidium bromide.^{14,15}

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Table 1. Sources and Characteristics of Pan-T MoAbs and Complement Used in This Study

MoAbs

First combination (Ortho)

CD3/OKT3 (IgG2a) = 0.2 μg to 1 $\mu\text{g}/1 \times 10^7$ NC

CD2/OKT11 (IgG2) = 0.7 μg to 1 $\mu\text{g}/1 \times 10^7$ NC

Second combination (A. Bernard¹³)

CD2/D66 (IgGM) }
CD5/A50 (IgG2a) } 10 $\mu\text{g}/\text{L}/10^7$ NC
CD7/I21 (IgG2b) }

Rabbit Complement

White baby rabbits (23 to 28 days)

Anti-A anti-B heterohemagglutinins negative

Hematopoietic stem cell recovery ($\geq 70\%$)

Hemolytic activity (CH50 = 29.6 units $\pm$ 6.9)

Calcium level = 3.06 $\pm$ 0.156 mmol/L

T cell lysis (standardized conditions with pan-T MoAbs)

Limulus test negative

Bacteriologic and virologic controls negative

Measurements indicate mean ± 1 SD.

Approximately 200 to 500 cells were examined and counted under a Leitz fluorescent microscope.

Bone marrow processing and ex vivo treatment. Bone marrow was aspirated from the iliac crests and from the sternum (2 mL per aspirate to avoid peripheral blood contamination). Our protocol has been previously reported^{11,16} and is summarized in Fig 1. This procedure takes approximately six hours and 30 minutes to complete.

Patients' characteristics. A total of 32 patients (18 males, 14 females; 19 adults, 13 children) were included in our open pilot study. The median age was 20 years (ranging from 2 to 40 years). Ten previously reported patients¹¹ are included in this group.

The diagnoses were: nine acute myelogenous leukemia (AML), 16 acute lymphocytic leukemia (ALL), five chronic granulocytic leukemia (CGL), and two multiple myeloma. Table 2 indicates the patients' status at the time of the BMT. All the patients were HLA-A, B, DR identical with their sibling donors. Mixed lymphocyte cultures were bidirectionally negative for all of them. There were seven major blood group incompatibilities. High risk of acute GVHD was expected in ten patients over 30 years of age, in two patients with CGL in acute or accelerated phase, and in four patients with acute leukemia in relapse or subsequent remission. Ten patients exhibited all the criteria of malignant disease with high risk, ie, major chromosome abnormalities in six patients, high WBC count ($\geq 50 \times 10^9/L$) in nine patients, first relapse on therapy in the course of an acute lymphoblastic leukemia (fast disease) in eight patients, and subsequent remission or relapse in four patients. The patients

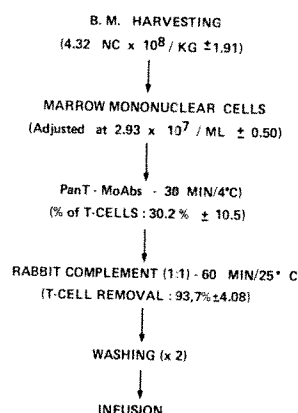


Fig 1. Bone marrow processing and ex-vivo treatment protocol. Measurements indicate mean ± 1 SD.

were conditioned by fractionated TBI (2 Gy for six fractions for a total dose of 12 Gy) preceding high dose of cyclophosphamide (60 mg per kg body weight × 2). Irradiation was delivered through a linear accelerator with an exposure rate of 5 to 6 cGy per minute. Protocol and consent forms were approved by the institutional review board of the University of Besançon.

Five ALL patients and one AML patient received reinforced chemotherapy in the pre-transplant regimen, according to their high risk of relapse (cytosine arabinoside 100 mg/m² for five days and/or etoposide 100 mg/m² per day for three days). In two patients high dose melphalan (180 mg/m²) was used as a substitute for cyclophosphamide. One multiple myeloma patient was given high-dose melphalan (140 mg/m²) combined with cyclophosphamide preceded by a fractionated TBI.

All but three patients were nursed in closed plastic tent isolators (Isroom, La Calhène, France) with a total nonselective gut decontamination using non absorbable antibiotics. All the patients were given low-dose heparin (1 mg/kg/d) for prophylaxis of veno-occlusive disease of the liver.¹⁷

During the initial feasibility study, posttransplant immunosuppression with methotrexate was maintained until day 100 in four patients and stopped at day 11 post-BMT in six patients (group 1). In 22 patients, no posttransplant immunoprophylaxis was administered (group 2). No maintenance chemotherapy was given following BMT. The median duration of isolation from BMT was 30 days (ranging from 17 to 49 days). Eighteen patients received bone marrow treated with the first MoAb combination (OKT-pool) and 14 with the second (CD-pool). The day of engraftment was defined as the third consecutive day on which the WBC count was $\geq 1.0 \times 10^9/L$. Acute GVHD is defined as beginning <90 days after transplant. Severity was defined according to the Seattle criteria.¹⁸ Genetic markers were available to prove engraftment in 25 patients (sex chromosomes in 12 patients and red blood cell phenotype disparities in 13 patients). No marker was available in seven patients. Chromosome studies were performed by direct processing of the unstimulated bone marrow specimens. An average of 16 mitoses (3 to 110) were analyzed in detail at three, six, and 12 months post-BMT.

Cellular immune function. Immune status was evaluated through surface cell markers by using a panel of seven specific T and B cell MoAbs, lymphocyte proliferative responses to mitogens (PHA, con A) and allogeneic cells (mixed lymphocyte culture). The periods tested were pretransplant and one, three, six, and 12 months posttransplant.

RESULTS

Marrow transplants for the study patients were performed between January 1983 and August 1985.

Early mortality. Of the 32 patients who underwent BMT, eight (five of whom were children) died within three months (mean 35.6 days) due to transplantation-related complications (six had ALL, two had AML). Early toxicity was significantly higher in the patients who had received additional chemotherapy in the preparative regimen (5/8). The causes of death were one fungal septicemia, three interstitial pneumonitis, one acute pancreatitis, one toxoplasmosis, one pseudomonas infection that localized in the neck, and one cardiac and renal failure. The patients who died within 21 days and 40 days post-BMT were not evaluable for engraftment of acute GVHD prevention. Thus 28 patients were evaluated for engraftment and 27 for acute GVHD prevention.

T cell depletion. Processing of the bone marrow led to

Table 2. Clinical Status, Engraftment Data and Outcome of 32 Patients Undergoing T Cell-Depleted Bone Marrow Transplantation

UPN	Age/sex	Diagnosis	Prognostic Factor	GVHD		Chromosome Analysis	Red Cell Antigens (%)	GVHD		Survival (Days)
				Prophylaxis Post-BMT	Engraftment			Acute	Chronic	
1	29/M	CGL (BT)	HR	MTX (long)	YES	4/4 Ph1 ⊖	NA	0	0	150 (relapse)
2	21/F	AML2 (CR2)	HR	MTX (long)	YES	NA	100	0	0	900+
3	37/M	CGL (AP)	HR	MTX (long)	YES	4/4 Ph1 ⊖	NA	0	1	860 + (2nd PC)
4	10/M	AML2 (CR2)	HR	MTX (long)	YES	NA	100	0	0	170 (relapse)
5	13/M	ALL1 (CR3)	HR	MTX (short)	YES	NA	100	0	0	810+
6	4/F	ALL1 (CR2)	HR	MTX (short)	YES	6/6*	NA	0	0	210 (relapse)
7	40/F	CGL (AP)	HR	MTX (short)	YES	3/3 Ph1 ⊖	100	0	1	630 + (2nd PC)
8	35/F	MULT MYELOMA	HR	MTX (short)	YES	NA	NA	0	NE	105 (relapse)
9	5/F	ALL1 (2nd rel)	HR	MTX (short)	YES	6/6*	NA	0	0	330 (relapse)
10	17/M	ALL1 (CR1)	SR	MTX (short)	YES	4/10*	NA	0	0	660 + (CR2)
11	29/M	AML5 (CR1)	HR	NO	YES	NE	NE		NE	19 (cardiac and renal failure, pneumonia)
12	12/F	ALL-T (2nd rel)	HR	NO	YES	NE	NE	0	NE	43 (interstitial pneumonitis)
13	38/M	ALL-T (CR2)	HR	NO	NE	NE	NE		NE	27 (interstitial pneumonitis)
14	28/F	AML2 (PR)	HR	NO	YES	NA	100	0	0	480 (relapse)
15	32/M	CGL (CP1)	SR	NO	YES	12/12* Ph1 ⊖	NA	1	0	450+
16	2/M	ALL2 (CR2)	HR	NO	NE	NE	NE		NE	11 (fungal septicemia)
17	2/M	ALL1 (CR2)	HR	NO	YES	3/10*	NA	0	0	105 (relapse)
18	9/M	ALL2 (CR2)	HR	NO	NE	NE	NE		NE	20 (acute pancreatitis)
19	32/F	AML1 (CR1)	SR	NO	YES	3/3*	100	1	0	240 (relapse)
20	20/M	ALL1 (CR2)	HR	NO	YES	NA	NA	0	0	270 (relapse)
21	6/M	AML5 (CR2)	HR	NO	YES	NA	100	0	0	90 (interstitial pneumonitis)
22	39/F	AML3 (CR1)	SR	NO	YES	28/28 (1qh ⁺ :neg)	100	0	0	310+
23	30/M	AML2 (CR1)	SR	NO	YES	NA	NA	0	0	276 (relapse)
24	8/F	ALL1 (CR2)	HR	NO	YES	110/110*	100	0	0	270+
25	15/F	ALL2 (CR2)	HR	NO	YES	NA	100	0	0	246+
26	18/M	T-ALL (CR1)	SR	NO	YES	12/12*	100	4	3	150 (GVHD)
27	5/M	AML2 (CR1)	SR	NO	YES	NA	NA	0	0	215+
28	38/F	CGL (CP1)	SR	NO	YES	9/9 Ph1 ⊖	100	2	1	180+
29	6/F	ALL1 (CR2)	HR	NO	NE	NE	NE		NE	8 (pseudomonas infection)
30	23/M	ALL2 (CR1)Ph1 ⁺	HR	NO	YES	NE	NA	0	0	210 (relapse)
31	18/F	ALL2 (CR1)	SR	NO	YES	12/12*	100	1	NE	60 (toxoplasmosis)
32	30/F	MULT MYELOMA	HR	NO	YES	NA	NA	2	1	140 (relapse)

Abbreviations: UPN, Unique patient number; HR, High risk; SR, standard risk; NA, Not available; NE, Not evaluable; CR, complete remission; CP, Chronic phase; AP, Accelerated phase; BT, Blastic transformation.

*Number of donor cells/number of evaluated cells by X/Y analysis.

the separation of marrow mononuclear cells on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), which was well adapted to a complement-mediated cytotoxicity. The harvested donor marrow volume was 470 mL $\pm$ 118 and contained a mean of $4.3 \pm 1.9 \times 10^8$ nucleated cells per kilogram recipient weight. These data demonstrate that it is not necessary to aspirate a large volume of bone marrow to harvest sufficient nucleated cells to ensure engraftment. The small volume of collected marrow avoided a perioperative blood transfusion. A density gradient separation of the marrow resulted in a $79\% \pm 16\%$ recovery of committed myeloid progenitors. Treatment with pan-T MoAbs and complement resulted in 1 to 1.5 log of T cell depletion from 30 study marrows out of 32.

The mean T cell depletion was $94\% \pm 4\%$. T cells

harvested with the transplant were $74 \pm 36 \times 10^7$. One round of complement lysis produced an incomplete T cell depletion. A total of $4.7 \pm 3 \times 10^7$ residual viable T cells remained in the inoculum, and the number of viable T cells infused to the patient were $0.99 \pm 0.65 \times 10^6$ per kilogram of the recipient's weight. Although the use of OKT-MoAbs led to a smaller cell clumping than the CD2, -5, and -7 pool, we did not observe a significant difference between the two MoAb combinations in terms of T cell depletion efficacy. The T cell removal with the OKT-pool and the CD-pool was $93\% \pm 6\%$ and $95\% \pm 3\%$ respectively. Committed stem cell assays done before and after complement incubation indicated a $66\% \pm 21\%$ CFU-GM recovery. These data are summarized in Table 3. In most patients the main side effects of the treated marrow infusion were chills and fever.

Table 3. T Cell Counts Before and After Ex-vivo Treatment in HLA-Matched Situation

N* T cells in the transplant $\times 10^7$	= 73.7% $\pm$ 36.1%
N* residual viable T cells $\times 10^7$	= 4.71% $\pm$ 3.27%
% of T cell depletion	= 93.7% $\pm$ 4.08%
1st MoAb association (OKT-pool)	= 93.17% $\pm$ 5.81%
2nd MoAb association (CD-pool)	= 94.85% $\pm$ 2.61%
T cell infused to the patient $\times 10^6/\text{kg}$	= 0.99% $\pm$ 0.65%
CFU-GM recovery post-T cell removal	= 69.2% $\pm$ 17.2%

Measurements indicate mean $\pm$ 1 SD.

*N = 32

Engraftment. Engraftment was achieved in all the study patients who received a T cell-depleted transplant. At the time of this analysis (October 15, 1985), no patients had an early (no engraftment within 30 days post-BMT) or a late (graft rejection beyond 90 days post-BMT) graft failure.

The time of recovery to $1 \times 10^9/\text{L}$ white blood cells was 14 to 28 days (mean 18.9 ± 4.7). For 65% of the patients the recovery time was under 20 days. Thirteen to 41 days were needed to recover $0.5 \times 10^9/\text{L}$ granulocytes (mean 20.5 ± 6.4). The time required was under 20 days for 54% of the patients. The time to recover $50 \times 10^9/\text{L}$ platelets was 16 to 65 days (mean 29.2 ± 12.5). The time required was under 30 days for 56% of the patients.

Proof of total chimerism was established in 20 patients by utilizing either genetic markers, eg, sex chromosomes in 11 patients or RBC antigen disparities in 13. Mixed chimerism was documented in two ALL patients grafted in first and second complete remission (CR). One patient relapsed 16 months after BMT; following this he achieved a second remission resulting from the German ALL-BFM-82 chemotherapy protocol.

Acute and chronic GVHD. Twenty-four of the 27 evaluable patients (88%) did not have an acute GVHD higher than grade 0 to 1. No acute GVHD occurred in the first group of ten patients. In group 2, two patients had grade 2 (skin only) confirmed by skin biopsy. Both responded promptly to 2 mg/kg/d of prednisone. A third patient developed a grade 4 GVHD. This patient received more viable T cells than the other patients because of unforeseen technical problems during the in vitro treatment. He was treated with two cycles of bolus intravenous (IV) high-dose methylprednisolone and rabbit antithymocyte globulins. Three months later evidence of extensive chronic GVHD developed in this patient, and he died four months posttransplant.

No other patient developed extensive chronic GVHD. Nevertheless, limited chronic GVHD occurred in four patients (Sicca syndrome in two patients, focal skin lesions in two others). These manifestations promptly disappeared after low-dose prednisolone. In 19 study patients the follow-up was longer than six months.

Chronic GVHD and infectious complications. It is well known that infectious complications that occur after the first three months are seen primarily in patients with a chronic GVHD.¹⁹ A majority of deaths attributed to late infections are reported in patients with active chronic GVHD. Seventeen study patients without extensive disease were analyzed

to evaluate if the clinically significant late infection rate was reduced. Most of them had either none or only one or 2 late infections. Twelve infectious episodes were documented in the 17 study patients (mean: 0.7 ranged between 0 and 3). Skin and genitourinary tract infections and septicemia were the most common types. One impressive example was that of our patient with an active chronic GVHD. He suffered six severe infectious episodes before death occurred.

Relapse. At the time of analysis a medullary leukemia relapse occurred in 13 patients (out of 24 evaluable patients), prior to six months in eight patients and after six months in five. Nine of the 13 were previously defined as "high risk leukemia." In this series the longest period between transplant and relapse was 364 days. Eleven patients have remained in unmaintained continuous CR to date. The mean follow-up is that of 13.3 months (ranging from three to 30 months) with only three patients below six months. The current status of the 32 study patients is reported in Table 4.

Immunologic reconstitution. Recovery of the immune function will be reported at length elsewhere (E. Racadot). In all engrafted patients there was considerable impairment of immunologic status after allogeneic BMT using incomplete T cell-depleted marrow (Fig 2). This severe immune deficiency persisted beyond one year posttransplant. These immune alterations are equivalent to those reported by Zander et al²⁰ after allogeneic BMT using untreated marrow.

DISCUSSION

The removal of T cells by pan-T MoAbs and complement-mediated cytotoxicity is a simple and effective method available in clinical situations. The cytolytic effect of these pan-T MoAbs depends closely on the source of complement and on the anticomplementary effect of bone marrow cells.²¹ Treatment with antibodies and complement in vitro can achieve an efficient and rapid killing of donor T cells. The extent of the removal of T cells can be accurately measured. We did not observe any difference between the two MoAb combinations used in terms of engraftment kinetics and GVHD prevention. The major finding of this study was that a prompt engraftment was achieved in all the patients who had received ex-vivo treated marrow. No early or late graft failure occurred in 17 patients beyond six months after BMT. Our results differ from the clinical data that have been published recently.^{7,10} Martin et al reported 20 patients grafted with marrow treated with a mixture of eight MoAbs and rabbit

Table 4. Current Status of the BMT Program With a T Cell-Depleted Inoculum

Number of patients	= 32
BMT related death (≤ 3 months)	= 8 (25%)
Number of take	= 28/28 EP
Late graft failure	= 0
Acute GVHD $\geq$ grade 2	= 3/27 EP (11.1%)
Chronic GVHD	= 1/19 EP (5.2%)
Relapse	= 13/24 EP (54.1%)
Number of living patients (disease free)	= 11/24 (45.8%)

EP = Evaluable patient.

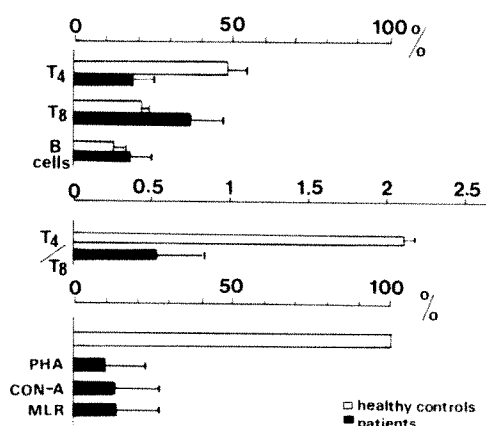


Fig 2. Summary of immune profile in patients grafted with T cell-depleted marrow six months after BMT.

complement that led to a 2- to 3-log depletion of T cells. Eight cases of graft failure occurred, seven of them being among the 11 patients prepared for transplantation with 12.0 Gy of TBI, and only one out of the nine patients was prepared with 15.75 Gy.⁷

As part of a European cooperative study on T cell depletion with Campath-1 MoAb (IgM, which fixes human complement and binds both T and B lymphocytes), the incidence of graft failure was high while varying from one team to another (10% to 20%).²² In a prospective randomized controlled trial, clinical data from the UCLA Center showed that five of 20 patients in the CT2/CD2-treated marrow group failed to engraft (none were in the control group).²³ The association of graft failure with a near complete removal of T cells from the donor marrow suggests that T cells in the marrow graft may eliminate host effector cells that survive the conditioning regimen.

Our clinical results on engraftment strongly suggest that our own conditioning regimen protocol may play a major part in the patient's immunologic status. Engraftment depends closely on the magnitude of the residual host-v-graft reactivity caused by residual host T cells, which might react against donor antigens exhibited on donor hematopoietic stem cells.²⁴ Some recent data suggest that increased immunosuppression can overcome the risk of graft failure.⁷

An experimental model is under study in our laboratory in an attempt to demonstrate the influence on the immunologic status of the host at the time of BMT in relation to radiation exposure of the recipient. We would like to emphasize that the patients who received fractionated TBI a week before grafting tolerated the preparative regimen far better.

Evidence shows that acute GVHD can be effectively prevented by a depletion of T cells from the bone marrow. Our own results are in agreement with the recently published results of the Royal Free Hospital,⁵ the Fred Hutchinson Center,⁷ the University of Wisconsin,²⁵ and the Hamman-Smith Hospital.¹⁰ The difference lies at the T cell removal level. A $\geq 99\%$ T cell depletion is reported in most publications, whereas in our experience a mean of 94% T cell depletion has been observed. It could be suggested that a near-to-complete removal does not appear necessary to pre-

vent severe forms of acute GVHD. Nevertheless, we have to interpret our results with caution given the relatively small group of evaluable patients in group 2.

The outlook is even more promising insofar as chronic GVHD is concerned. Extensive chronic GVHD occurred in only one of the 22 study patients followed beyond three months. It is obvious that the late infection rate decreased significantly. To date, we can assume that, independently of its source, a pan-T MoAb cocktail is highly effective in preventing severe forms of acute and chronic GVHD, even in the absence of posttransplant immunoprophylaxis. Other MoAbs in combination with the pan-T MoAb can be used for a T cell depletion, such as antibodies that recognize the CD4 and CD8 T cell molecules.

The suppression of severe forms (grade 3 to 4) of acute GVHD and of extensive forms of chronic GVHD should improve the patient's survival rate in the first year posttransplant, although this fact has not been demonstrated in the present study. Nevertheless, we have to point out that the quality of life is excellent given the low frequency of late infections; the Karnovsky score has always been higher than 90%. We must not, however, overlook the fact that a graft-v-leukemia effect has been closely associated with acute and especially chronic GVHD. Such close correlations were clearly shown when the allograft was done in the course of second remission or later.²⁶ One may therefore expect to find that an improvement in the survival rate is associated with an increase in the relapse rate. However, the clinical series reported by Prentice et al⁵ on a homogeneous group of acute leukemia patients who had undergone a BMT in CR1 did not show that the relapse rate had increased. Nevertheless, we may wonder if a total GVHD prevention in high-risk leukemia is reasonable when the BMT is done in CR2 and subsequent remission. It is hoped that some light will be shed on this question once the randomized clinical trials in progress are completed.

In conclusion, the results of this open pilot study lead us to assume that it has been possible to prevent GVHD successfully through a T cell depletion alone and to obtain engraftment in all the study patients who did not have a late graft failure. Although an evaluation is premature at this stage, the relapse rate appeared to be relatively high in our series. Perhaps there is a close connection with the high percentage of study patients defined as "high risk leukemic."

NOTE ADDED IN PROOF

The authors report two graft rejections and one graft failure in a new series of 14 T-depleted matched BMT since the time of the analysis reported above.

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Chlorpropamide-Induced Pure White Cell Aplasia

By Lee J. Levitt

We investigated the mechanism for isolated agranulocytosis and marrow pure white cell aplasia in an elderly man receiving 0.5 to 1.0 g per day of chlorpropamide (Chl) without other toxic drug exposure or overt systemic illness. Patient marrow revealed an absence of recognizable granulocytic precursors; megakaryocytes and erythroid precursors were normal. The WBC count was 1800/mm³ on admission with only 2% neutrophils; the absolute neutrophil count first exceeded 500/mm³ on the 17th day following cessation of Chl. A serum Chl level on admission was 100µg/mL (acute phase, AP); no Chl was detected in serum (convalescent phase, CP) assessed on the 22nd hospital day. Antineutrophil antibodies were not detected, and T cell depletion failed to augment patient in vitro granulopoiesis. Patient AP serum produced potent complement-mediated inhibition (87% ± 7%) of autologous granulocyte progenitors (CFU-GM) with minimal inhibition of

erythroid (11% ± 5%) or multipotent (5% ± 4%) progenitor cells. Selective inhibition by patient AP serum of CFU-GM (74% ± 11%) was also seen against two allogeneic marrows. Patient CP serum no longer inhibited (6% ± 4%) autologous CFU-GM. Addition of Chl (5 to 120 µg/mL) to CP serum but not to control serum resulted in potent drug concentration-dependent complement-mediated inhibition of autologous and allogeneic CFU-GM. Inhibition of CFU-GM in the presence of Chl was no longer demonstrable following immunoabsorbent removal of IgG from patient serum. Patient serum in the presence of Chl had limited activity against morphologically recognizable marrow granulocytic precursors in a microimmunofluorescence assay. These results are most consistent with the development of Chl-dependent, selective antibody-mediated immune inhibition of granulopoiesis.

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IMMUNE-MEDIATED drug-dependent destruction of mature neutrophils has been well documented as a cause of agranulocytosis.^{1,2} Development of techniques for the in vitro clonogenic assay of hematopoietic progenitors has also permitted investigation of clinical syndromes associated with immunologic injury to human stem cells.^{3,4} Drug-induced inhibition of human myeloid progenitor growth has been reported in patients with drug-associated neutropenia,^{5,7} and spontaneously arising (non-drug-associated) antibodies directed against granulocyte-macrophage progenitor cells have also recently been described.⁸ The mechanism of drug-dependent suppression of granulopoiesis, however, has not always been clearly elucidated, and the cellular and antigenic specificity of antibodies mediating myeloid stem cell injury have not been well characterized. Failure to demonstrate serum inhibitory activity against autologous granulocyte progenitors in many of these reports also raises questions about the possible alloimmune nature of the antibodies and the pathogenic roles of these inhibitors in the development of neutropenia.

We have previously reported the occurrence of spontaneously arising selective autoantibody-mediated immune inhibition of granulopoiesis in a patient with agranulocytosis

and profound marrow granulocytic hypoplasia (primary pure white cell aplasia).⁸ In this report we investigated the mechanism for acquired neutropenia and marrow pure white cell aplasia in an elderly man receiving high doses of chlorpropamide (Chl) with no other history of toxic drug exposure or overt systemic illness.

CASE REPORT

An 81-year-old man was admitted to the hospital in May 1985 for fever and severe neutropenia. A routine hemogram performed in March 1985 had demonstrated a normal WBC count. The patient was subsequently admitted to Stanford Hospital with fever, otalgia, and neutropenia. He had a prior history of adult onset (Type II) diabetes mellitus treated with dietary restriction and for the last three months had taken 500 to 1,000mg PO daily of Chl. The patient was also taking occasional acetaminophen and oxycodone, and there was no family history of blood dyscrasias. On physical examination he was febrile with a left otitis externa. Phimosis and balanitis were present. The nodes, spleen, and liver were not palpable, and the remainder of the examination was unremarkable. Routine serum chemistries were normal except for a creatinine of 2.7mg/dL. The hematocrit was 35%, the platelets were 486,000/µL, and the WBC count was 1,800/µL with 2% neutrophils, 93% lymphocytes, 3% monocytes, and 2% eosinophils. Direct and indirect (with and without 100µg/mL Chl) Coombs' tests were negative. The reticulocyte count was 1.0%. A bone marrow aspirate and biopsy revealed a mild decrease in overall cellularity (35%). There was virtually a complete absence of granulocytes and all morphologically recognizable myeloid precursors. Erythroid maturation was normal, and megakaryocytes appeared increased in number. No evidence for a thymoma was detected on chest roentgenograms. The patient had a normal serum B₁₂ and folate level and an unremarkable serum immunoelectrophoresis. A serum Chl level obtained on the second hospital day was 100µg/mL (average plasma Chl concentrations following ingestion of 250 mg; range from 19-114 µg/mL, with the variation most dependent upon differences in drug half-life and variations in the volume of distribution). Chlorpropamide was not detectable when the level was again assessed on the 22nd hospital day. The patient was begun on tobramycin and cefoperazone, and his Chl was discontinued. His fever gradually defervesced over the ensuing seven days with a gradual improvement in his balanitis and otitis. The patient's absolute neutrophil count slowly increased and first exceeded 500/µL on the 17th hospital day. A repeat bone

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marrow aspirate performed on the 25th hospital day demonstrated reconstitution of marrow myeloid precursors with normal maturation and a myeloid:erythroid (M:E) ratio of approximately 2:1.

METHODS

Assay systems for hematopoietic progenitors. Two to four milliliters of marrow aspirate from normal adult bone donors were drawn into heparinized syringes, layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradients, and interface mononuclear cells collected. All donors gave fully informed consent prior to marrow aspiration, and all studies were approved by the Stanford University Human Experimentation Committee. Marrow mononuclear cells (5 to $15 \times 10^4/\text{mL}$) were cultured in the presence or absence of 0.5 IU of purified human urinary erythropoietin ($1,140$ U/mg of protein, National Institutes of Health) in triplicate 0.25 -mL aliquots in methylcellulose microwell cultures containing 1% Mo-conditioned medium (MoCM, a medium conditioned by a T lymphoblast cell line containing both myeloid colony-stimulating activity and erythroid burst-promoting activity^{9,10}). Committed erythroid progenitors (burst-forming units-erythroid [BFU-E]) and granulocyte-macrophage progenitors (colony-forming units-granulocyte-macrophage [CFU-GM]), as well as multipotent hematopoietic stem cells (colony-forming units giving rise to mixed colonies containing granulocytic, erythroid, macrophage, and megakaryocytic cells [CFU-GEMM]) were enumerated by in situ observation after 10 to 14 days of incubation at 37°C as previously described.^{8,11} Marrow CFU-GM were also independently assessed in cultures containing 15% human placental conditioned medium, in place of MoCM, as a source of myeloid colony-stimulating activity.¹² In some experiments marrow cells were further fractionated by double-adherence monocyte depletion (less than 2% residual monocytes) and/or solid-phase immunoabsorption with pan-T Leu-1 monoclonal antibody to deplete T lymphocytes (less than 1% to 2% residual marrow T cells) as previously described.¹¹

Cytotoxicity and immunoabsorption studies. Antibody and drug-dependent complement-mediated cytotoxicity against human hematopoietic progenitors was assessed as previously described.⁸ Suspensions of 2×10^6 nonadherent buoyant marrow cells per milliliter were prepared in Iscove's modified Dulbecco's medium. Test serums (patients acute [day 2] and recovery [day 25] serum or control AB serum) were preincubated with saline or diluent controls or varying concentrations of Chl (8 to $120\mu\text{g}/\text{mL}$ final concentration) for 30 minutes at 37°C . Chlorpropamide (Diabinese, Pfizer Pharmaceuticals, New York) was dissolved in either 100% ethanol or in 0.15 mol/L sodium hydroxide with heating and subsequent neutralization with 0.15 mol/L hydrochloric acid; appropriate diluent controls were used in all experiments. One quarter milliliter of the drug-serum mixture was incubated with an equal volume of the marrow cell suspension with or without tested rabbit complement (Pel-Freeze Biological, Rogers, AR) for 30 to 60 minutes at 37°C . After incubation, marrow cells were washed twice, and aliquots were plated in methylcellulose. No decreases in colony formation were noted after cell incubation with complement, control human AB serum, or drug diluent alone at appropriate final concentrations. In some experiments, marrow cells, patient or control serum, drug suspension, and fresh rabbit complement were combined and incubated together at 37°C for 60 minutes prior to washing and plating. In other experiments, drug suspensions were preincubated with marrow cells with or without control sera for 30 minutes at 37°C ; the cells were then washed twice and incubated with test serums with or without a source of fresh complement prior to plating.

Serum antineutrophil activity was independently assessed by a modified granulocyte-microagglutination technique¹³ and by a granulocyte cytotoxicity assay¹⁴ against a panel of granulocytes from ten

normal donors in the presence and in the absence of 20 to $100\mu\text{g}/\text{mL}$ chlorpropamide. Patient antineutrophil activity was also assessed by a Cowan I staphylococcal slide test; the latter includes both direct and indirect tests for detection of cell-bound antibody and allows direct visualization of small numbers of autologous granulocytes during periods of severe granulocytopenia.¹⁵ A fluorochromatic modification of the Terasaki micromethod was used to assay for lymphocyte cytotoxicity against a panel of lymphocytes from 19 normal donors.¹⁶

Patient serum activity against morphologically recognizable marrow granulocytic precursors was assessed via an indirect immunofluorescence technique as previously described⁸; marrow cell pellets were treated with test serum with or without varying concentrations of Chl (20 to $110\mu\text{g}/\text{mL}$ final concentration) or with 80H.5 monoclonal antibody (an IgM complement-fixing murine hybridoma antibody specifically recognizing an antigen on mature granulocytes and on immature marrow myeloid precursor cells¹⁷). Following incubation at 4°C for 20 minutes, cell suspensions were washed with phosphate-buffered saline and were then incubated with either goat antihuman fluorescein isothiocyanate (FITC)-labeled anti-IgG antibody or goat antimouse FITC-labeled anti-IgM antibody (Tago, Burlingame, CA) prior to examination by phase and fluorescence microscopy.

Absorption of IgG from test serums was performed using protein A-sepharose CL-4B affinity chromatography before and after treatment with either rabbit or goat anti-IgG.⁸ Absorbed samples were assessed for adequacy of separation by immunoelectrophoresis; virtually all IgG in the absorbed serums was removed by the immunoabsorbent procedure. Patient's serum was also absorbed with pooled platelets from 14 random donors to remove possible class I (but not class II) anti-HLA alloantibodies as previously described.^{8,18}

RESULTS

Patient marrow obtained on the first and third days of hospitalization was cultured separately in methylcellulose to enumerate marrow multipotential (CFU-GEMM) and committed granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitor cells. Patient colony yield per 2×10^5 buoyant marrow cells included 158 ± 26 (mean $\pm$ SEM) BFU-E, 67 ± 9 CFU-GM, and 4.0 ± 1.8 CFU-GEMM. These results are comparable to those obtained from prior and/or concurrent culture of ten normal human marrows in our laboratory (Table 1). Colony cytologic composition, examined after micropipette selection and histochemical staining, did not differ between patient and control marrows after assessment of individual CFU-GM and CFU-GEMM from each experiment. Independent assessment of patient CFU-GM, using placental-conditioned medium rather than Mo-conditioned medium as a source of myeloid colony-stimulating activity, yielded colony numbers comparable to those obtained after plating of normal human marrow. No increment in patient marrow CFU-GM was seen following marrow T cell depletion by immunoabsorption using a pan-T Leu-1 monoclonal antibody (data not shown). These data together indicate that neither a deficiency of marrow granulocyte-macrophage or multipotent progenitors nor qualitative changes in patient colony cytologic composition could explain the patient's agranulocytosis and marrow white cell aplasia. These results also provide no evidence for autologous T cell inhibition of granulopoiesis.

Table 1. Inhibitory Effect of Patient Acute-Phase Serum on Growth of Autologous Marrow Hematopoietic Progenitors

	Colonies/ 2×10^5 Cells			Percent CFU-GM Inhibition
	CFU-GM	BFU-E	CFU-GEMM	
Control BM*	55 $\pm$ 8	145 $\pm$ 22	3.5 $\pm$ 2.0	—
Patient BM†	67 $\pm$ 9	158 $\pm$ 26	4.0 $\pm$ 2.8	—
Patient BM + AB Serum + C'†	63 $\pm$ 7	143 $\pm$ 19	3.2 $\pm$ 2.0	6
Patient BM + Patient Serum†	59 $\pm$ 81	149 $\pm$ 23	3.0 $\pm$ 1.7	12
Patient BM + Patient Serum + C'†	9 $\pm$ 4	141 $\pm$ 18	3.7 $\pm$ 2.5	87

*Mean $\pm$ SEM from ten separate experiments, each plated in duplicate in methylcellulose cultures.

†Mean $\pm$ SEM from two separate experiments, each plated in triplicate in methylcellulose cultures. Mo-conditioned medium was used as a source of myeloid colony-stimulating activity in both patient and control cultures. Marrow was obtained on two separate occasions during the first five days of hospitalization while the patient was still neutropenic.

BM, bone marrow; C', complement; CFU-GM, colony-forming units for granulocytes and macrophages; BFU-E, burst-forming units-erythroid; CFU-GEMM, colony-forming units for granulocytes, erythroid cells, macrophages, and megakaryocytes.

Patient AP serum was next examined for antineutrophil activity. Both the direct and the indirect Cowan I staphylococcal slide tests performed to detect antineutrophil antibodies were negative. Serum granulocyte cytotoxicity and granulocyte agglutinating activity were assessed against a panel of ten normal donors. Neither the cytotoxicity nor the microagglutination assay detected antineutrophil activity in patient serum. The patient's acute and chronic-phase sera were cytotoxic for lymphocytes derived from only three of 19 normal blood donors.

We next assessed patient AP serum (obtained on the second hospital day) for its effect on autologous marrow hematopoietic progenitors. In two separate experiments using MoCM as a source of myeloid colony-stimulating activity, patient heat-inactivated serum in the presence of complement produced potent (mean 87%) inhibition of autologous marrow CFU-GM with minimal inhibition of either BFU-E or CFU-GEMM (Table 1). Similarly, patient AP serum produced potent complement-mediated inhibition of autologous marrow CFU-GM when patient granulocyte macrophage progenitors were cultured in the presence of placental conditioned medium (Fig 1). The further addition of as much as 120 μ g/mL of Chl to patient AP serum was not associated with any further significant decrement in autologous marrow CFU-GM. Similar selective inhibition of CFU-GM was noted against two separate allogeneic human marrows after treatment with patient serum plus complement (Fig 1). Patient serum did not demonstrate cytotoxicity for lymphocytes derived from either of the two allogeneic marrow donors. Minimal inhibition (12% to 19%) of allogeneic BFU-E or CFU-GEMM was noted after treatment of marrow with patient serum plus complement. Patient serum in the absence of complement had little inhibitory effect on allogeneic hematopoietic progenitors.

The effect of patient acute (day 2, containing 100 μ g/mL Chl) and convalescent (day 25, containing no detectable Chl)-phase serum with and without exogenous Chl was next assessed on growth of autologous marrow CFU-GM. Patient marrow was obtained for this set of experiments upon recovery of the WBC count to 6,800 per μ L, four weeks following cessation of Chl. Previously frozen patient AP serum in the presence of complement again selectively inhibited autologous marrow CFU-GM (Fig 2). However, patient convalescent-phase serum in the presence of complement

failed to inhibit either autologous or allogeneic marrow granulocyte-macrophage progenitors. Patient convalescent-phase serum in the presence of Chl (60 μ g/mL final concentration) did inhibit (79% $\pm$ 21%) autologous CFU-GM (Fig 2). Inhibition of autologous CFU-GM by patient convalescent-phase serum was seen when the previously prepared drug-serum mixture was incubated with marrow plus complement. Inhibition was also noted when marrow, serum, drug suspension, and complement were incubated together simultaneously (88% $\pm$ 26%). Autologous CFU-GM inhibition was not seen, however, when the drug suspension was preincubated with marrow, after which marrow cells were washed and then incubated with serum plus complement prior to plating (11% $\pm$ 7%). No inhibition was noted after marrow cell incubation with AB serum plus 60 μ g/mL Chl plus complement (Fig 2).

Inhibition of patient CFU-GM colony growth by convalescent-phase serum was drug-concentration dependent (Fig 3)

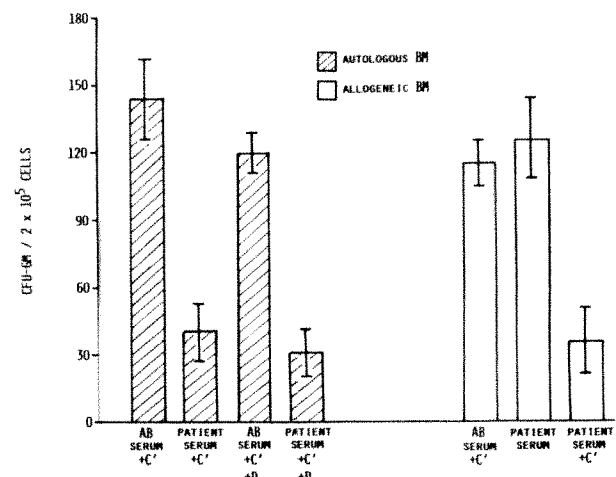


Fig 1. Effects of patient AP serum (containing 110 μ g/mL Chl) with or without exogenous Chl (120 μ g/mL) on growth of autologous and allogeneic granulocyte-macrophage (CFU-GM) progenitor cells. C', complement; D, chlorpropamide. Results expressed as means $\pm$ SEM from two to three separate experiments. Patient acute-phase serum produced potent, C'-mediated inhibition of both autologous and allogeneic marrow CFU-GM. Patient marrow was obtained on days one and three of hospitalization while the patient was still neutropenic.

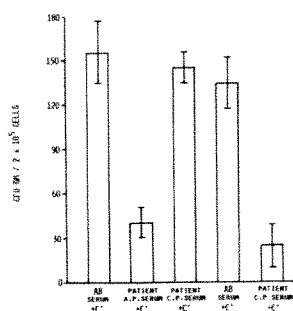


Fig 2. Effects of patient AP (containing 110 $\mu\text{g}/\text{mL}$) and convalescent-phase (containing no detectable levels of Chl) serum with or without exogenous Chl (60 $\mu\text{g}/\text{mL}$ final concentration) on growth of autologous marrow granulocyte-macrophage (CFU-GM) progenitor cells. C', complement; D, chlorpropamide. Results expressed as means $\pm$ SEM from two separate experiments. Patient convalescent-phase serum inhibited growth of autologous CFU-GM only in the presence of exogenous drug. Marrow was obtained from the patient following recovery of neutropenia.

with demonstrable progenitor inhibition occurring at Chl concentrations of 30 to 120 $\mu\text{g}/\text{mL}$ (the patient's AP serum Chl level on the second hospital day was 100 $\mu\text{g}/\text{mL}$). Patient serum plus Chl in the absence of complement had little demonstrable inhibitory activity against autologous CFU-GM (Fig 3). Patient convalescent-phase serum plus Chl with or without complement had little inhibitory activity against autologous BFU-E.

Absorption studies of patient serum were next performed utilizing protein A-sepharose affinity chromatography to deplete patient serum of IgG immunoglobulin. As demonstrated in Fig 4, inhibition of allogeneic CFU-GM in the presence of 100 $\mu\text{g}/\text{mL}$ Chl could no longer be demonstrated following immunoabsorbent removal of IgG from patient convalescent-phase serum. Absorption of patient's serum with pooled platelets from 14 random donors to remove possible anti-HLA antibody activity did not affect the inhibitory activity of patient convalescent-phase serum plus Chl against allogeneic CFU-GM.

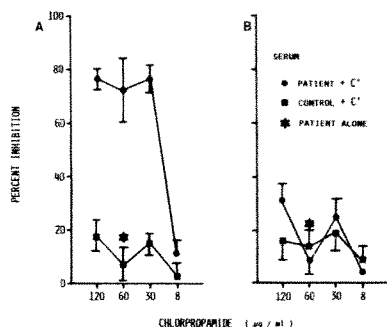


Fig 3. Effects of patient convalescent-phase serum plus varying amounts of exogenous Chl on growth of autologous marrow granulocyte-macrophage (CFU-GM, A) and early erythroid (BFU-E, B) hematopoietic progenitor cells. Marrow was obtained following recovery of neutropenia. C', complement. Results expressed as means $\pm$ SEM from two separate experiments. Patient serum plus drug produced selective drug concentration-dependent inhibition of allogeneic CFU-GM.

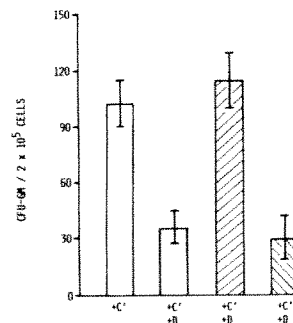


Fig 4. Effects of Chl (100 $\mu\text{g}/\text{mL}$ final concentration) plus immunoabsorbed convalescent-phase patient serum on growth of allogeneic granulocyte-macrophage (CFU-GM) progenitor cells. C', complement; D, chlorpropamide. CFU-GM inhibition was no longer demonstrable following protein A immunoabsorbent removal of IgG from patient serum. Pooled platelet immunoabsorption of patient serum to remove possible anti-HLA alloantibodies had no effect on CFU-GM inhibition. Results expressed as means $\pm$ SEM from two to three separate experiments. $\square$, Patient serum; $\square$, patient serum after protein A immunoabsorption; $\square$, patient serum after pooled platelet immunoabsorption.

Patient serum was next assessed for activity against morphologically recognizable marrow myeloid precursors utilizing an indirect immunofluorescent assay. Results were compared with similar assays on the same allogeneic target marrow population using an antihuman IgM monoclonal antibody termed 80H.5. This antibody recognizes an antigen common to mature granulocytes and immature marrow granulocytic cells and was positive by immunofluorescence for 54% $\pm$ 9% of nonadherent allogeneic marrow mononuclear cells. This percentage corresponded to the number of morphologically recognizable granulocytic precursors as assessed by Wright-Giemsa staining of marrow target cell cytocentrifuged preparations. Patient convalescent-phase serum in the presence or absence of 100 $\mu\text{g}/\text{mL}$ Chl was positive for 9% $\pm$ 3% of allogeneic marrow cells ($n = 3$). These results were not different from those obtained after incubation with allogeneic marrow and AB serum with or without Chl (11% $\pm$ 4%). The results suggest that patient serum in the presence or absence of Chl had limited immunofluorescent activity against morphologically recognizable allogeneic marrow granulocytic precursors.

DISCUSSION

This patient's severe neutropenia and marrow pure white cell aplasia completely resolved following discontinuation of Chl and in the absence of immunosuppressive therapy. Patient AP serum (containing 100 $\mu\text{g}/\text{mL}$ Chl) produced potent complement-mediated inhibition of both autologous and allogeneic granulocyte-macrophage (CFU-GM) hematopoietic progenitors with little demonstrable cytotoxic activity against autologous or allogeneic erythroid (BFU-E) and multipotent (CFU-GEMM) progenitors. Patient serum also possessed little activity against autologous and allogeneic lymphocytes, granulocytes, or morphologically identifiable marrow granulocytic precursors. Patient convalescent-phase serum (obtained after recovery of marrow and peripheral

blood granulocytopenia and four weeks following cessation of Chl) no longer inhibited autologous or allogeneic CFU-GM. However, the addition of Chl to patient convalescent-phase serum resulted in potent drug concentration-dependent, complement-mediated selective inhibition of autologous and allogeneic CFU-GM. Inhibition of allogeneic CFU-GM was no longer demonstrable following protein A-sepharose immunoabsorbent removal of IgG from patient serum. These results are most consistent with the development of Chl-dependent selective antibody-mediated immune inhibition of granulopoiesis.

The etiology and pathogenesis of marrow pure white cell aplasia appear in many ways analogous to the previously described syndrome of acquired pure red cell aplasia.^{19,20} Primary chronic pure red cell aplasia has been associated in about 50% of cases with IgG autoantibodies causing either complement-dependent erythroblast lysis, complement-independent inhibition of erythroid progenitor colony growth, or, rarely, inhibition of endogenous circulating erythropoietin.²¹⁻²⁴ We have previously described a patient with pure white cell aplasia associated with a spontaneously arising (non-drug-dependent) complement-fixing IgG autoantibody that selectively inhibited autologous and allogeneic CFU-GM proliferation.⁸ Thymomas have also been reported in association with both acquired pure red cell aplasia and with acquired marrow granulocytic hypoplasia.^{21,25} At least four cases of Chl-associated pure red cell aplasia have been previously reported.²⁶⁻²⁹ Agranulocytosis has also been described as a rare complication of Chl administration; marrow smears in these patients have revealed varying degrees of hypocellularity and only partial preservation of erythroid elements and megakaryocytes.^{30,31} Stein et al have described a female patient with fatal agranulocytosis associated with Chl therapy and the presence of serum leukoagglutinins. The marrow in this case was described as hypocellular with "profound depression of myeloid cells" but with normal erythroid cells and megakaryocytes.³² No further studies were undertaken to evaluate the mechanism of the selective marrow myeloid hypoplasia.

A number of mechanisms have been postulated to explain the pathogenesis of drug-induced immune cytotoxicity.³³ In penicillin-induced hemolytic anemia, the drug is tightly bound to erythrocytes *in vitro* and *in vivo*³⁴; inhibition of autologous CFU-GM was not seen in our patient, however, when Chl was preincubated with target marrow mononuclear cells prior to washing and incubation with patient serum plus complement, thus making a drug-adsorption mechanism unlikely. An "innocent bystander" mechanism has been described in a case of Chl-induced immune hemolytic anemia in which the causal antibody was found to be a complement-requiring IgG immunoglobulin.³⁵ Alternatively, drugs may in some patients lead to the production of "autoantibodies" directed against intrinsic red cell antigens.³³ In this instance, however, antibodies are usually demonstrable in the absence of drug, and cell cytotoxicity usually persists for several weeks to months following cessation of drug therapy.

The explanation regarding cellular specificity of drug-induced immune cytotoxicity remains to be elucidated. Recent

evidence suggests, however, that target cells may have receptors for some drugs or drug-antibody complexes.³⁶ Chlorpromamide-induced immune hemolytic anemia has been described in at least one instance to be associated with an antibody directed against Jk^a antigenic determinants.³⁷ Human red blood cell surface antigenic determinants have not, however, been found on hematopoietic progenitor cells.³⁸ Claas et al have provided evidence that certain drugs may induce autoantibody formation by reacting with restricted non-HLA polymorphic determinants present on the surface of either platelets or granulocytes.³⁹ Cellular specificity in drug-antibody reactions may also be conferred as a consequence of alternative mechanisms of drug-antibody binding to target cell determinants: binding of antidrug antibody to target cells, which occurs only in the presence of drug, has been shown in preliminary experiments utilizing quinidine-dependent antiplatelet antibodies to be mediated by the Fab domain of the antibody rather than the Fc portion of the immunoglobulin.⁴⁰ Our data seem most consistent with the hypothesis that binding of patient drug-antibody complex occurs via an antigenic determinant or receptor that is relatively restricted in expression to the surface of human CFU-GM and which appears to be lost during the early stages of progenitor cell differentiation.⁴¹

Granulocyte-macrophage progenitors, obtained from the patient during a time of profound neutropenia, grew normally in the absence of patient serum or drug. These observations suggest that the assayed colonies may have arisen from primitive precursors that did not bind the patient's drug-antibody complex. Erythroid colonies are often similarly present in normal or increased numbers in the marrow of patients with antibody-mediated inhibition of erythropoiesis and pure red cell aplasia.^{23,42} Marrow colony growth in patients with humoral inhibitors of autologous hematopoiesis has also been described in spontaneously arising pure white cell aplasia and in patients with antibody-mediated aplastic anemia.^{8,43} Normal growth of patient CFU-GM may, alternatively, reflect differences *in vivo* and *in vitro* in the kinetics of drug-antibody receptor binding and cytotoxicity.¹³

Drug-associated inhibition of granulocyte-macrophage progenitor growth has been described infrequently in patients with agranulocytosis.^{5-7,44} Bone marrow smears, however, in a number of these patients have demonstrated coexistent suppression of erythroid and megakaryocytic activity or, alternatively, considerable preservation of myeloid activity with a relative paucity of only the more mature granulocytic elements. In some cases of purported drug-induced suppression of marrow colony formation, the drug inhibited growth of normal allogeneic myeloid colonies but did not suppress growth of patient recovery marrow colonies.^{5,7} Tactile et al have described two patients with diphenylhydantoin-associated antibody-mediated inhibition of granulopoiesis; sera from these patients, however, also appeared capable of mediating destruction of polymorphonuclear leukocytes.⁴⁵ Kelton et al have reported a patient with quinidine-induced pancytopenia and marrow hypoplasia; quinidine plus patient serum inhibited growth of both autologous and allogeneic erythroid and granulocytic progenitor

cells.⁴⁶ Very recently Mamus et al⁴⁷ have described the development of reversible marrow granulocytic aplasia in association with ingestion of ibuprofen. Inhibition of in vitro granulopoiesis in this instance was observed only in the presence of patient serum or IgG plus ibuprofen plus complement.

Further investigations of patients with drug-induced immune suppression of hematopoiesis should provide a better understanding of the mechanisms underlying drug-induced myelosuppression and may identify either surface antigenic determinants or antibodies which would prove useful in the recognition and purification of early hematopoietic progenitor cells.

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Altered Plasma Membrane Phospholipid Organization in *Plasmodium falciparum*-Infected Human Erythrocytes

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The intraerythrocytic development of the malaria parasite is accompanied by distinct morphological and biochemical changes in the host cell membrane, yet little is known about development-related alterations in the transbilayer organization of membrane phospholipids in parasitized cells. This question was examined in human red cells infected with *Plasmodium falciparum*. Normal red cells were infected with strain FCR3 or with clonal derivatives that either produce (K^+) or do not produce (K^-) knobby protuberances on the infected red cells. Parasitized cells were harvested at various stages of parasite development, and the bilayer orientation of red cell membrane phospholipids was determined chemically using 2,4,6-trinitrobenzene sulphonic acid (TNBS) or enzymatically using bee venom phospholipase A_2 (PLA $_2$) and sphingomyelinase C (SMC). We found that parasite development was accompanied by distinct alterations in the red cell membrane transbilayer distribution of phosphatidylcholine (PC), phos-

phatidylethanolamine (PE), and phosphatidylserine (PS). Increases in the exoplasmic membrane leaflet exposure of PE and PS were larger in the late-stage parasitized cells than in the early-stage parasitized cells. Similar results were obtained for PE membrane distribution using either chemical (TNBS) or enzymatic (PLA $_2$ plus SMC) methods, although changes in PS distribution were observed only with TNBS. Uninfected cohort cells derived from mixed populations of infected and uninfected cells exhibited normal patterns of membrane phospholipid organization. The observed alterations in *P. falciparum*-infected red cell membrane phospholipid distribution, which is independent of the presence or absence of knobby protuberances, might be associated with the drastic changes in cell membrane permeability and susceptibility to early hemolysis observed in the late stages of parasite development.

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MALARIA IS the world's most common form of hemolytic anemia and remains a major public health problem in many areas of the world. In 1983 the number of malaria cases reported worldwide was 5.5 million,¹ and it is estimated that approximately 65% of the world's population lives in malarious areas.²

Involvement of red cell membrane glycoproteins has been implicated in the attachment of parasites to the membrane,³⁻⁸ and parasite maturation has been shown to result in a redistribution of spectrin,⁹ the major membrane skeletal protein. Alterations in the morphology of the infected cells as well as changes in their osmotic fragility,¹⁰⁻¹³ membrane deformability,^{14,15} and permeability to cations,¹⁶ amino acids,¹⁷ and glucose¹⁷ strongly suggest that membrane lipids are also affected by parasitization.

In normal human erythrocytes, the phospholipids comprising the plasma membrane are distributed asymmetrically between the bilayer leaflets.¹⁸ This asymmetric distribution results in the exoplasmic leaflet being enriched in phosphatidylcholine (PC) and sphingomyelin (SM) while the cytoplasmic leaflet is enriched in phosphatidylethanolamine (PE) and contains all of the phosphatidylserine (PS). Alterations in the bilayer distribution of membrane phospholipids have been noted in several pathologic erythrocytes including sickle cells,¹⁹ spherocytes due to spectrin deficiency,²⁰ elliptocytes due to protein 4.1 deficiency,²¹ as well as in erythrocytes undergoing drug-induced endocytosis.²² We have investigated the organization of membrane phospholipids in human red cells infected with knobby and knobless strains of *Plasmodium falciparum* and determined that significant changes occur in their bilayer distribution during the intracellular maturation of the parasites.

MATERIALS AND METHODS

Cultivation of *P. falciparum*. Parasites were grown at a 5% cell suspension by the method of Jensen and Trager.²³ The FCR3 strain was maintained in synchronous development by a combination of sorbitol treatment²⁴ and gelatin enrichment,²⁵ as proposed by Kile-

jian.²⁶ Sorbitol treatment resulted in cultures highly enriched (>98%) in "early ring" stage-infected cells. Gelatin enrichment resulted in cultures that were predominantly (>75%) late-stage parasites (trophozoites and schizonts). Knobby (A2,K⁺) and knobless (D4,K⁻) clones of FCR3²⁷ were synchronized with sorbitol treatment only. The extent of parasitemia and stage of development was determined by Giemsa-stained cells.

For experiments using the FCR3 strain of *P. falciparum*, fresh, washed normal human red cells were inoculated with late schizonts obtained from gelatin supernatants (parasitemia > 80%) to a final parasitemia of 5% and plated at 1% cell suspension prior to incubation.²⁸ For experiments using the K⁺ or K⁻ clones of FCR3, cultures were first grown at a 1% cell suspension to a parasitemia of >30%. This material was diluted to 5% parasitemia with fresh, washed red cells and plated as for FCR3. For controls, uninfected red cells from the same donor as the infected samples were incubated simultaneously.

Late ring-infected cells were collected from culture approximately 24 hours after inoculation. Trophozoite-infected cells were collected at approximately 36 hours and schizont-infected cells at approximately 48 hours. In some experiments FCR3 trophozoite- or schizont-infected cells were enriched from uninfected red cells by gelatin treatment.²⁵

Determination of erythrocyte plasma membrane phospholipid

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organization. Red cell membrane phospholipid organization was determined enzymatically using phospholipase A₂ (PLA₂, from bee venom) and sphingomyelinase C (SMC, from *Staphylococcus aureus*) or chemically using the amino-labeling reagent trinitrobenzene sulphonic acid (TNBS). In the absence of extensive cell hemolysis, these probes do not penetrate the membrane bilayer and react only with those phospholipids exposed on the red cell membrane surface. Therefore, the extent to which the various classes of phospholipids react with these probes is a measure of their exposure in the membrane exoplasmic leaflet.

At various stages of parasite development, 0.1 mL washed packed red cells were treated with 12.5 or 25 units PLA₂ at 37 °C for 45 minutes followed by 1.5 or 3 units SMC for 30 minutes, or with 5 mmol/L TNBS for 1 hour in a total volume of 4 mL (final hematocrit of 2%). The buffer used for the PLA₂/SMC experiments contained 10 mmol/L sodium phosphate, pH 7.4, 10 mmol/L sodium chloride, 0.25 mmol/L magnesium chloride, 0.5 mmol/L calcium chloride, 130 mmol/L potassium chloride and 10 mmol/L glucose; while that used for the TNBS experiments contained 50 mmol/L sodium phosphate, pH 8.5, 1.2 mmol/L potassium phosphate, 120 mmol/L sodium chloride, 5 mmol/L potassium chloride, 0.5% bovine serum albumin, and 5.5 mmol/L glucose. Following the incubation the cells were collected by centrifugation, and the extent at cell hemolysis was determined by the supernatant absorbance of 540 nm. Enzyme-treated cells were immediately lysed with 0.5 mL 5 mmol/L EDTA (pH 7.4), while TNBS-treated cells were washed once in the incubation buffer (pH 7.4), twice in 0.9% saline (pH 7.4), and then lysed with 0.5 mL water. The lipids were extracted, individual phospholipid classes separated by thin-layer chromatography,²⁹ and quantitated by phosphorous determination.³⁰ The percentage of phospholipid hydrolysis (for enzyme-treated cells) or modification (for TNBS-treated cells) was calculated as the ratio of the amount of hydrolysed (modified) phospholipid to the amount of total (hydrolysed or modified + native) phospholipid for each phospholipid species.

Hemoglobin electrophoresis. Aliquots of the red cell lysates prepared from the control or TNBS-treated cells were subjected to hemoglobin electrophoresis on cellulose acetate strips.³¹ Each sample contained the equivalent of from 5 to 10 μ L packed cells; the electrophoresis was performed at pH 8.5 at 380 V for 20 minutes at 24 °C in a Helena Electrophoresis Chamber (Helena Labs, Beaumont, TX). The electrophoresed cellulose acetate strips were stained in Ponceau S stain [0.5% (wt/vol) Ponceau S, 3.5% (wt/vol) sulfosalicylic acid, 3.5% (wt/vol) trichloroacetic acid] and then destained in 5% acetic acid.

RESULTS

Plasma membranes from normal uninfected human erythrocytes maintained in cell culture for periods of time equivalent to those of the *P. falciparum*-infected cells (24 to 48 hours) contained approximately 32% PC, 28% PE, 14% PS, and 25% SM. The total amount of extractable membrane phospholipid in the late-stage parasitized cell populations was about 20% greater than in the uninfected control or early-stage parasitized cells. This increase is likely due to presence of a parasitophorous membrane that encapsulates the parasites and develops during parasite maturation. Membranes from infected cells contained similar proportions of membrane phospholipids, suggesting that parasitization and development of the parasitophorous membrane was not accompanied by conversion of one phospholipid species to another (Table 1). Subjecting normal uninfected erythro-

cytes to phospholipid hydrolysis using PLA₂ and SMC yielded the pattern given in Table 2. Combined PLA₂ and SMC treatments yielded hydrolysis of 75% $\pm$ 3% of the PC, 27% $\pm$ 2% of the PE, none of the PS, and 59% $\pm$ 1% of the SM. In contrast to uninfected cells, *P. falciparum*-infected cells containing early-stage (ring-form) parasites showed a moderate increase in PE hydrolysis (33% $\pm$ 6%, $P < 0.001$) with a concomitant decrease in PC hydrolysis (57% $\pm$ 5%, $P < 0.001$). Hydrolysis of SM (54% $\pm$ 6%) was not significantly different from that of the uninfected cells (59% $\pm$ 1%), nor was there any hydrolysis of PS. Intracellular maturation of the parasites to trophozoites and schizonts resulted in further increases in PE hydrolysis (46% $\pm$ 6%, $P < 0.001$). Infected cells containing clonal derivatives producing knobs (K⁺) also showed marked elevations in PE hydrolysis that progressively increased as the parasites matured from early-stage forms to late-stage forms (34% $\pm$ 8% and 44% $\pm$ 7%, respectively, $P < 0.001$). Similarly, infected cells containing clonal derivatives lacking knobs (K⁻) exhibited increased PE hydrolysis (42% $\pm$ 2% in the late-stage forms, $P < 0.001$, not measured in the early-stage forms). Decreases in the extent of PC hydrolysis were also observed in both the K⁺- and K⁻-infected cells, similar to those seen in the uncloned FCR3-infected cells. Membrane organization of SM was examined only in the early-stage parasitized cells where no differences were observed, nor was there any evidence for PS hydrolysis in any of the infected cells. As noted in Table 1, maturation of the parasites resulted in an approximate 20% increase in total extractable membrane phospholipid.

Although our data indicate that parasitized cell membranes demonstrate a loss of phospholipid asymmetry, we can only speculate on whether such changes represent a complete loss of asymmetry in subpopulations of the infected cells or a partial loss of asymmetry in all of the infected cells. To address this issue we performed calculations using the data given in Table 2 and compared the calculated values to the measured values for phospholipid hydrolysis. For example, considering PE hydrolysis in the synchronous cell culture (Syn)-infected cells, calculations assuming 100% PE hydrolysis (ie, complete loss of PE asymmetry) in all of the parasitized cells yielded an average value of 40% PE hydrolysis. This calculated value compares favorably with the measured value of 46% $\pm$ 6% PE hydrolysis by PLA₂ and SMC in the Syn-late-stage infected cells. Calculations assuming only 50% PE hydrolysis in all of the parasitized cells yielded an average of 31% PE hydrolysis. This calculated value agrees closely with the measured value of 33% $\pm$ 6% PE hydrolysis in the Syn-early-stage infected cells. Similarly, calculations for PE hydrolysis in the K⁺ and K⁻-infected cells yielded an average of 36% PE hydrolysis if all of the PE had translocated to the exoplasmic leaflet and 30% if only 50% of the PE had translocated. These calculated values agree favorably with the measured values of 44% $\pm$ 7% and 34% $\pm$ 8% for K⁺-late- and K⁻-early-stage infected cells, respectively. Thus, our calculations suggest but do not prove, that in early-stage parasitemia only a portion (~50%) of cytoplasmic leaflet PE becomes accessible to the exoplasmic leaflet, whereas in late-stage parasitemia essentially all of

Table 1. Plasma Membrane Phospholipid Content of Human Erythrocytes Infected with *P. falciparum*

Erythrocyte	% Parasitemia	Phospholipid Content, % of Total Phospholipids				Total PL
		PC	PE	PS	SM	
Normal erythrocytes maintained in cell culture	0	32 ± 2	28 ± 2	14 ± 1	25 ± 3	1.8 ± 0.2
Syn-early stage (rings)	17	32 ± 3	26 ± 3	14 ± 2	27 ± 4	1.8 ± 0.3
Syn-late stage (trophozoites + schizonts)	18	33 ± 1	25 ± 3	13 ± 2	27 ± 3	2.2 ± 0.3*
K ⁺ -early stage	14	32 ± 3	25 ± 2	13 ± 3	27 ± 4	1.7 ± 0.1
K ⁺ -late stage	13	31 ± 1	24 ± 2	14 ± 2	27 ± 3	2.0 ± 0.4*
K ⁻ -early stage	13	34 ± 4	26 ± 2	13 ± 1	27 ± 3	1.8 ± 0.1
K ⁻ -late stage	12	35 ± 3	28 ± 3	13 ± 2	24 ± 4	2.1 ± 0.3*

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were incubated for 75 minutes at 37 °C in buffer containing 10 mmol/L sodium phosphate, pH 7.4, 10 mmol/L sodium chloride, 0.25 mmol/L magnesium chloride, 0.5 mmol/L calcium chloride, 130 mmol/L potassium chloride and 10 mmol/L glucose. Following the incubation the cells were collected by centrifugation, and the extent of cell hemolysis was determined from the supernatant absorbance at 540 nm. The cells were then lysed with 0.5 mL 5 mmol/L EDTA, the lipids were extracted, individual phospholipid species separated by thin-layer chromatography and quantitated by phosphorous determination. Erythrocytes infected with *P. falciparum* strain FCR3 were maintained in synchronous cell culture (Syn) as were the cells infected with clonal derivatives that produce (K⁺) or do not produce (K⁻) knobby protuberances. Early stage parasites were predominantly ring forms, while late stage parasites were predominantly trophozoite and schizont forms. Results for total extractable membrane phospholipid (Total PL) are given as µg phosphate/0.1 mL packed cells. Results presented for each phospholipid species represent the percent of total phospholipids, and are the mean (± 1 SD) of from three to eight determinations.

*Significantly different from uninfected normal erythrocytes at $P < 0.05$.

the cytoplasmic leaflet PE becomes accessible to the exoplasmic leaflet.

An alternative method for detecting phospholipids exposed on the surface of cells involves chemically labeling the aminophospholipids using 2,4,6-trinitrobenzene sulphonic acid (TNBS). This reagent only slowly penetrates living cells and has been used by many laboratories to covalently label aminophospholipids exposed on the surface of both normal³²⁻³⁴ and pathologic erythrocytes.³⁴ We obtained similar results using TNBS as we did using phospholipases. As shown in Table 3, uninfected cells maintained in cell culture exhibited labeling of both PE (9% ± 5%) and PS (2% ± 2%). The extent of PE labeling increased dramatically in the parasitized cells; the uncloned FCR3 ring-form parasitized cells showed a threefold increase in PE labeling

($P < 0.001$) that continued to increase as the parasites matured to trophozoites (fivefold increase, $P < 0.001$). Similar increases in PE labeling were also seen in the K⁺ and K⁻ clonal derivatives; however, the extent of increase (two- to threefold, $P < 0.001$) was about 30% less than that seen in the FCR3-infected cells. This may be due to the lower levels of parasitemia achieved in the clonal derivatives or may represent a selective effect of cloning. The extent of PS labeling showed a slightly different pattern from that of PE labeling; none of the early stage parasitized cells showed labeling of PS above uninfected cell values regardless of whether the parasites were of the FCR3, K⁺, or K⁻ variants. However, cells infected with late (trophozoite)-stage parasites showed small but statistically significant increases in PS labeling ($P < 0.05$ for K⁺-trophozoites, $P < 0.003$ for K⁻-tro-

Table 2. Plasma Membrane Phospholipid Organization of Human Erythrocytes Infected with *P. falciparum*

Erythrocyte	% Parasitemia	Phospholipid Hydrolysed, % of Total Phospholipids				Total PL
		PC	PE	PS	SM	
Normal erythrocytes maintained in cell culture	0	75 ± 3	27 ± 2	0	59 ± 4	1.8 ± 0.2
Syn-early stage (rings)	17	57 ± 5*	33 ± 6*	0	54 ± 6	1.8 ± 0.3
Syn-late stage (trophozoites + schizonts)	18	50 ± 4*	46 ± 6*	0	ND	2.2 ± 0.3†
K ⁺ -early stage	14	62 ± 9*	34 ± 8*	0	57 ± 4	1.7 ± 0.1
K ⁺ -late stage	13	55 ± 6*	44 ± 7*	0	ND	2.0 ± 0.4†
K ⁻ -early stage	13	62 ± 7*	ND	0	52 ± 2	1.8 ± 0.1
K ⁻ -late stage	12	51 ± 9*	42 ± 2*	0	ND	2.1 ± 0.3†

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were treated with 25 units bee venom phospholipase A₂ for 45 minutes at 37 °C followed by three units *Staphylococcus aureus* sphingomyelinase C for 30 minutes at 37 °C. Erythrocytes infected with *P. falciparum* strain FCR3 were maintained in synchronous cell culture (Syn) as were the cells infected with clonal derivatives that produce (K⁺) or do not produce (K⁻) knobby protuberances. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was < 6%.

Results for total extractable membrane phospholipid (Total PL) are given as µg phosphate/0.1 mL packed cells.

Values represent the mean (± 1 SD) of from three to eight determinations. Significantly different from normal erythrocytes at $P < 0.001$,* or $P < 0.05$,†

ND = Not determined.

Table 3. Plasma Membrane Phospholipid Organization of Human Erythrocytes Infected with *P. falciparum*

Erythrocyte	% Parasitemia	% Phospholipid Labeled by TNBS		Total PL
		PE	PS	
Normal erythrocytes maintained in cell culture	0	9 ± 5	2 ± 2	1.8 ± 0.2
Syn-rings	18	24 ± 3*	2 ± 3	1.8 ± 0.3
Syn-trophozoites	17	41 ± 8*	2 ± 1	2.0 ± 0.4
Syn-schizonts	20	43 ± 6*	7 ± 2*	2.2 ± 0.4†
K ⁺ -rings	14	16 ± 1*	3 ± 3	1.7 ± 0.1
K ⁺ -trophozoites	13	30 ± 7*	4 ± 1†	2.0 ± 0.4†
K ⁻ -rings	13	17 ± 2*	2 ± 1	1.8 ± 0.1
K ⁻ -trophozoites	12	29 ± 4*	5 ± 1‡	2.1 ± 0.3‡

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were treated with 5 mmol/L TNBS for 1 hour at 37 °C. FCR3-parasitized cells were maintained in synchronous cell culture (Syn) as were the cells infected with clonal derivatives that produce (K⁺) or do not produce (K⁻) knobby protuberances. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was < 5%.

Results for total extractable membrane phospholipid (Total PL) are given as µg phosphate/0.1 mL packed cells.

Values represent the mean (± 1 SD) of from eight to 12 determinations. Significantly different from normal erythrocytes at $P < 0.001$, * $P < 0.05$, † or $P < 0.003$ ‡.

phozoites); this increase was most marked in the schizont-infected cells (fourfold increase, $P < 0.001$). Increases in PE and PS labeling were not associated with any increases in cell hemolysis, which was always < 5%.

It should be noted that our measurements given in Tables 1 to 3 were made using mixtures of both infected and uninfected (cohort) cells. Since < 20% of the cells contained parasites, it is likely that the relatively small changes in phospholipid organization we found for PS in these cell mixtures actually represented much larger changes in the parasitized cells, assuming that cohort cells were unaffected. To address this issue directly we separated cohort cells from parasitized cells by sorbitol treatment²⁴ and examined their membrane phospholipid organization using phospholipases. As shown in Table 4, we found no evidence to indicate that membrane phospholipid organization was abnormal in cohort cells. To minimize hemolysis during cell treatment we used smaller amounts of enzymes in the cohort cell experiments than in the experiments reported in Table 2; therefore, the values obtained for cohort cell membrane phospholipid hydrolysis were somewhat lower.

It is obvious that the validity of using either enzymatic hydrolysis or chemical modification of phospholipids to probe for the exoplasmic leaflet content of phospholipids depends on the reagents being restricted to exoplasmic leaflet phospholipids. Although the extent of cell hemolysis is the

most often used indicator of an intact (and thereby impermeable to the probe) cell membrane, it is certainly not the most accurate method, since even small amounts of penetration by the probes could result in the hydrolysis or modification of cytoplasmic leaflet phospholipids. To more accurately determine the extent of probe penetration in our assay system, we made use of the fact that TNBS covalently labels both phospholipids and proteins containing primary amines. Penetration of intact cells by TNBS would, therefore, result in the labeling of hemoglobin. TNBS-labeled hemoglobin is more negatively charged than is native hemoglobin and can be separated and quantitated by standard hemoglobin electrophoresis techniques. When we analyzed hemolysates prepared from the TNBS-treated cells, we did not find evidence for hemoglobin modification unless the cells were first lysed prior to the addition of TNBS (Fig 1). It can be argued that a TNBS-hemoglobin adduct might not be visible electrophoretically if only a small percentage of the added TNBS actually penetrated the cell membrane. Therefore, to evaluate the sensitivity of our system for detecting TNBS-hemoglobin, we incubated normal uninfected erythrocyte hemolysates (made from a 2% hematocrit) with varying concentrations of TNBS (0 to 5 mmol/L) and examined the electrophoretically separated hemoglobins for the presence of TNBS-hemoglobin. We found that at TNBS concentrations of > 0.25 mmol/L, complete modification of hemoglo-

Table 4. Plasma Membrane Phospholipid Organization of Uninfected Cohort Populations from *P. falciparum*-Infected Human Erythrocytes

Erythrocyte	% Parasitemia	Phospholipid Hydrolysed, % of Total Phospholipids				Total PL
		PC	PE	PS	SM	
Normal erythrocytes maintained in cell culture	0	64 ± 1	14 ± 2	0	26 ± 2	1.7 ± 0.2
Uninfected cohort cells from Syn-late-stage infected mixed cell populations	< 1	72 ± 8	15 ± 1	0	31 ± 7	1.8 ± 0.2

Erythrocytes (0.1 mL packed cells, 2% final hematocrit) were treated with 12.5 units bee venom phospholipase A₂ for 45 minutes at 37 °C followed by 1.5 units *Staphylococcus aureus* sphingomyelinase C for 30 minutes at 37 °C. Uninfected erythrocytes from mixed populations of erythrocytes infected with late-stage forms of strain FCR3 maintained in synchronous cell culture (Syn) were separated by sorbitol treatment. Membrane phospholipids were separated and quantitated as in Table 1. Treatment-induced cell hemolysis was < 3%.

Results for total extractable membrane phospholipid (Total PL) are given as µg phosphate/0.1 mL packed cells.

Values represent the mean (± 1 SD) of four determinations.



Fig 1. Hemoglobin pattern obtained with cellulose acetate electrophoresis. Parasitized cells (2% final hematocrit) were reacted with 5 mmol/L TNBS, as in Table 3, after which the cells were washed three times with saline, hemolysates were made in water, and electrophoresis was performed. Lane 1, erythrocytes infected with trophozoites; lanes 2 to 4, erythrocytes infected with schizonts; lanes 5 to 6, uninfected erythrocytes; lane 7, TNBS-reacted erythrocyte lysate; lane 8, hemoglobin standards (C,S,F,A). Or = Sample application origin.

bin to TNBS-hemoglobin occurred, as evidenced by the complete disappearance of the unmodified hemoglobin band following electrophoresis (Fig 2). Thus, penetration of >5% of TNBS added to our experiments with intact cells (5 mmol/L) would have resulted in the complete modification of hemoglobin to TNBS-hemoglobin. The absence of detectable TNBS-hemoglobin in the TNBS-treated cells strongly suggests that TNBS did not penetrate the cell membrane to any appreciable extent, ie, that only those aminophospholipids located on the exoplasmic bilayer leaflet were labeled.

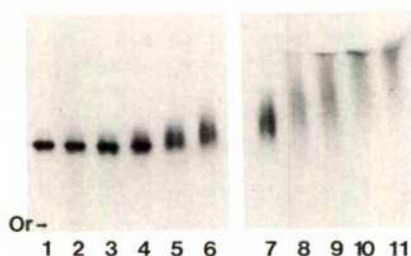


Fig 2. Hemoglobin pattern obtained with cellulose acetate electrophoresis. Hemolysates prepared from normal uninfected erythrocytes (2% final hematocrit) were reacted at pH 8.5 with TNBS (lane 1, no TNBS; lane 2, 0.05 mmol/L; lane 3, 0.1 mmol/L; lane 4, 0.25 mmol/L; lane 5, 0.5 mmol/L; lane 6, 0.75 mmol/L; lane 7, 1 mmol/L; lane 8, 2 mmol/L; lane 9, 3 mmol/L; lane 10, 4 mmol/L; lane 11, 5 mmol/L) for 1 hour at 37 °C, and then subjected to electrophoresis, as in Fig 1. The presence of TNBS-hemoglobin is indicated by the appearance of a diffuse band that migrates ahead of the unmodified hemoglobin along with the concomitant disappearance of the unmodified hemoglobin. Or = Sample application origin.

DISCUSSION

The evidence presented here suggests that infection and intraerythrocytic maturation of the malaria parasite *Plasmodium falciparum* is associated with profound alterations in the bilayer distribution of phospholipids in the infected host cell membrane. Using two different probes (enzymatic and chemical) to study red cell membrane bilayer phospholipid organization, we found that parasitization resulted in a partial loss of the normal asymmetric organization of membrane phospholipids as evidenced by the appearance of increased amounts of phosphatidylethanolamine (PE) and phosphatidylserine (PS) and a decreased amount of phosphatidylcholine (PC) in the exoplasmic leaflet of infected cells. The extent of these alterations was dependent upon the stage of parasite development; cells infected with early (ring-form) stages showed a partial redistribution of PE and PC but not PS, whereas cells infected with late (trophozoite- and schizont-form) stages also showed the above plus a partial redistribution of PS. Uninfected cohort cells that were cultured along with the infected cells exhibited normal patterns of membrane phospholipid organization. Other workers have similarly reported that cohort cells do not display membrane abnormalities. For example, Sherman and Greenan,³⁵ who examined membrane fluidity in *P. falciparum*-infected human erythrocytes, found no evidence for abnormalities in the uninfected cohort cells.

Redistribution of membrane phospholipids was found to occur in the FCR3 as well as in the knobby (K^+) and knobless (K^-) variants, suggesting that the appearance of knobby protuberances in the infected cells is not associated with specific alterations in the bilayer distribution of membrane phospholipids. Since only the K^+ strain is capable of venous sequestration,³⁶ we conclude that phospholipid redistribution is not involved in the interaction of parasitized cells with endothelium. This conclusion is supported by other workers who have reported that alterations in the phospholipid organization of human erythrocyte membranes does not enhance their adherence to endothelium.³⁷ However, increased exoplasmic leaflet content of aminophospholipids, in particular PS, has been shown to stimulate the adherence of affected red cells to mononuclear phagocytes.³⁸ Whether or not alterations in membrane phospholipid organization in parasitized red cells affect this aspect of the pathophysiology of the disease remains speculative; however, parasitized cells are known to be sequestered in large numbers by the spleen³⁹ and to undergo increased erythrophagocytosis⁴⁰ and adherence to autologous macrophages.¹⁴

Whereas we were able to detect increases in the exoplasmic leaflet content of PE using both the enzymatic (PLA₂ and SMC) and chemical (TNBS) probes, increases in the exoplasmic leaflet content of PS were only apparent using TNBS. Differences in the sensitivity of the probes for phospholipids could be due to the different region of the phospholipid molecule to which each probe reacts; the phospholipases react with the fatty acid-glycerol ester bond that is presumably buried within the hydrophobic core of the bilayer, whereas TNBS labels the aminophospholipid head group that is presumably exposed on the bilayer surface. Similarly, differences in probe sensitivity may be responsible

for the different extents of exoplasmic leaflet PE measured using TNBS and phospholipases in the cultured uninfected erythrocytes.

Other investigators have similarly reported that the plasma membrane of *P falciparum*-infected red cells exhibits abnormal properties, including increases in membrane disordering (fluidity)^{35,41} and a rearrangement of intramembraneous particles.⁴² In this regard, Gupta and Mishra⁴³ reported a redistribution of membrane phospholipids in rhesus monkey red cells infected with *Plasmodium knowlesi*. These authors observed large increases in the exoplasmic leaflet content of PE along with large decreases in the content of PC in that bilayer leaflet. In contrast to our results for *P falciparum*-infected human red cells, *P knowlesi*-infected monkey red cells did not exhibit any alteration in the bilayer distribution of PS. Such differences could be due to differences between monkey and human red cells, species differences between *P knowlesi* and *P falciparum*, or the different intraerythrocytic stages of infection examined (Gupta and Mishra examined only ring-stage-infected cells where we similarly did not find a redistribution of PS). However, the fact that infection of human and monkey red cells by different species of *Plasmodium* both resulted in the bilayer redistribution of membrane phospholipids strongly suggests that phospholipid reorganization may be a common event during the course of the intraerythrocytic maturation of malaria parasites. Alterations in the organization of phospholipids in the parasitized cell membranes could result in many of the observed membrane abnormalities, including

enhanced permeability to cations and nutrients,¹⁷ and may represent a membrane whose integrity is compromised as a result of modifications in membrane lipid-lipid or lipid-protein interactions. This loss of red cell membrane integrity could be a significant component in the early hemolysis of infected cells, a necessary requisite for the continuation of the intraerythrocytic asexual portion of *P falciparum*'s life cycle.

Although we did not specifically examine the mechanism by which phospholipid redistribution occurs in parasitized cells, it is likely to involve alterations in the rate of phospholipid translocation between bilayer leaflets. In this regard the bilayer translocation of PC in normal human erythrocytes is a relatively slow process, whereas the rate is dramatically increased in sickled erythrocytes.⁴⁴ It is also noteworthy that sickle erythrocytes exhibit increased exoplasmic leaflet amounts of PE and PS.^{19,34} Thus an increased interaction of the phospholipid probes with PE and PS may not represent a static increase in the exoplasmic leaflet content of those phospholipids but rather an increased rate at which they are translocated between bilayer leaflets and, hence, become available to interact with the probes.

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Murine Malaria Decreases Hemopoietic Stem Cells

By Paul H. Silverman, John C. Schooley, and Lynn J. Mahlmann

The causes of anemia and immunosuppression, major outcomes of malaria, are not well established. This study was undertaken to investigate whether erythropoietin (EP) production is adequate and whether the hemopoietic stem cells (CFU-S) were affected during the course of infection. Groups of female Balb/c mice infected with *Plasmodium vinckei vinckei*, *Plasmodium berghei*, or *Plasmodium chabaudi adami* were exposed to five hours of simulated altitude equivalent to 22,000 ft. Plasma samples were collected for EP bioassay and radioimmunoassay (RIA). Using radioiron incorporation as an index of erythropoiesis, differences in response to infection with different species of plasmodia were observed. In general, decreases in erythropoietic activity were observed in bone marrow and

spleen as the infection progressed and continued to be depressed after apparent resolution of a nonlethal infection with *P. chabaudi adami*. Marrow from infected and control femurs were tested for CFU-S content using the spleen colony assay. The cellularity and CFU-S content of the femoral marrow decrease as the parasitemia increases. All three species of plasmodia stimulate EP production during peak parasitemias, indicating that adequate amounts of EP are available to the erythron during malarial infection. Depletion of CFU-S and probable lack of compensatory turnover of CFU-S may contribute to the disease characteristics of malaria.

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ANEMIA is a major complication of malaria and contributes to the morbidity and mortality effects of the disease. Numerous studies over the past 20 years have indicated that mechanisms of red cell destruction and autoimmune responses are by themselves inadequate to account for the severity and extent of anemia caused by malaria.¹⁻⁴

Another aspect of malaria infection concomitant with anemia is immunosuppression. Efforts to elucidate these mechanisms have been largely limited to clinical observations and studies that have focused on peripheral blood cells.⁵⁻⁹ These studies have tended to suggest that deficiencies in T lymphocytes occur and that splenic macrophages play a role in regulating immune hyporesponsiveness during malaria infection. Other workers have suggested that immunosuppression is due either to impairment of the progenitor cells of granulocytes/macrophages or an imbalance of the competition between erythropoietic and immune cell lineages, which are all derived from a common pluripotent stem cell (CFU-S).^{10,11}

We¹² and others^{13,14} have observed that during infection with murine malaria, an ample quantity of erythropoietin (EP) is produced; however, erythroid progenitors fail to respond to the hormone. From the data presented here, which expands our previous report,¹² it is apparent that the CFU-S compartment in the bone marrow decreases during the course of infection.

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MATERIALS AND METHODS

Female Balb/c and outbred Swiss Webster mice (Bantin and Kingman, Fremont, CA) between 6 and 10 weeks of age were used as indicated in the text. The principles of laboratory animal care as promulgated by the Public Health Service National Institutes of Services were observed. The animals were housed in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility.

Three species of murine malaria were used: *Plasmodium vinckei vinckei* (Pvv), *P. berghei* (Pb), and *P. chabaudi adami* (Pca). Normal mice were inoculated by intravenous (IV) injection with 10⁵ infected red blood cells. Parasitemia counts were made from tail blood smears stained with Dif Quik (American Scientific Products, McGraw Park, IL). The percent of infected erythrocytes was determined by examining 200 to 1,000 red blood cells. Multiple infection of red cells with parasites were often observed but were recorded as a single infection.

Infected and uninfected Swiss Webster mice were exposed to five hours in a decompression chamber at a simulated altitude of 22,000 ft (6,706 m Po₂ = 67.1 torr) and then sacrificed. Infected and uninfected mice not exposed to simulated high altitude served as controls. Heparinized whole blood from groups of 12 to 14 mice was collected by cardiac puncture, pooled, and centrifuged. The plasma samples were dialyzed, lyophilized, and reconstituted to the original volumes. Erythropoietin levels were measured by a polycythemic assay^{15,16} and radioimmunoassay (RIA).¹⁷

Groups of six infected and uninfected BALB/c mice were injected IV with 0.5 μ Ci ⁵⁹FeCl₃ (New England Nuclear, Boston; catalogue no. NEZ-037) and sacrificed 6 hours later. The spleens and both femurs from each mouse were counted in a Hewlett Packard gamma well counter (Palo Alto, CA). One group of uninfected mice was injected intraperitoneally (IP) with phenylhydrazine (60 μ g/g) for two consecutive days. Two days after the second injection 0.50 μ Ci ⁵⁹FeCl₃ was given IV.

Recipient Balb/c mice for CFU-S bioassay were irradiated with 700 cGy (100 cGy/min) delivered from a ⁶⁰Co irradiation source just prior to transplantation.¹⁸ At this level of whole body irradiation there is less than 0.1 CFU-S per spleen in each group of ten endogenous irradiation control mice. These controls were routinely a part of the experimental protocol. Single cell suspensions of donor femoral marrow were prepared and injected IV into the recipients. Femoral cellularity was determined using Zap-oglobin II and a Coulter Counter model ZBI (Coulter Electronics, Hialeah, FL). Eight days later whole blood was collected via cardiac puncture for determining parasitemias and xenodiagnosis. Xenodiagnosis was performed by injecting IV 0.20 mL whole blood into normal mice

whenever the parasitemias of the CFU-S assay mice appeared negative. The spleens were placed in Bouins solution, and the number of spleen colonies were counted using a dissecting microscope. Some spleens were processed for histologic examination.

RESULTS

Erythropoietin production. The results summarized in Table 1 indicated that the plasma EP concentrations are normal during the early course of infection with all three species of plasmodia. When parasitemias are high (12% to 40%), EP concentrations increase two- to 100-fold, depending upon the species of *Plasmodium*. An additional challenge of hypoxia produces no additional EP in mice infected with Pb, but mice infected with Pvv or Pca do increase their plasma levels of EP.

Radioiron incorporation. Studies on the erythropoietic activity of the bone marrow and the spleen utilizing radioiron incorporation revealed a substantial increase in marrow and a decrease in the spleen of mice infected with Pvv (Table 2). Mice infected with Pb or Pca showed a dramatic decrease in erythropoiesis in both the marrow and spleen as the infection progressed to acute levels. During the late stages of infection with Pca when the parasitemia was very low or negative, radioiron incorporation was about 85% and 50% of normal in marrow and spleen, respectively (Table 3).

Bone marrow cellularity. Determinations of the total number of nucleated cells in the femora of mice infected with Pvv or Pb indicated consistently that a marked reduction occurs soon after infection (Table 4). The bone marrow cellularity of femora of mice continued to fall as the infection

progressed, dropping to 40% with Pvv and to 60% of normal with Pb shortly before death (Figs 1 and 2) ($P < 0.001$).

The total bone marrow cellularity of femora from mice infected with nonlethal Pca decreased to 60% of normal on days 7 to 9 postinoculation but began to recover several days after the peak parasitemia on day 12 (Fig 3, Table 4). It was observed that femora and other bones from mice in the later stages of murine malaria were brittle. It appeared that osteoclasia was taking place, resulting in thinner bones and an increase in bone marrow volume.

Spleen colony assay. During the process of transplantation of bone marrow cells for spleen colony assays, the possibility of transfer of parasites from infected donors to irradiated recipients was monitored by blood smear examination of the recipients on the day of sacrifice. In the majority of recipients no evidence of parasitemia was observed. In those recipient mice in which parasites were detected, the infection usually was present in trace amounts ($<1:1000$ RBC) and rarely 1% to 5% with Pvv. Histologic examination of spleens from recipient mice that received marrow from control or malaria infected donors revealed no differences in cell types or parenchymal colony distribution. Also, there were no apparent macroscopic differences or significant variations in the number of spleen colonies in individual mice in similar treatment groups with or without low-level malaria infections.

Stem cell changes. Infection with Pvv did not appear to increase the stem cell content in the femora during the early phase of the disease. Death from Pvv infection occurred seven to nine days after inoculation when the stem cell populations were reduced by more than 50% (Fig 1) ($P < 0.001$). During the early phases of infection with Pb, an increase in the CFU-S content of the femora was observed. However, in the late stages of infection with Pb stem cell content dropped to less than 25% of normal just before death of the host (Fig 2).

Pca infection had little or no effect on the stem cell content in the femora until the peripheral blood parasitemia was 15% as observed on day 7. However, the CFU-S per 10^6 nucleated bone marrow cells showed a 20% to 50% increase above normal during the early phase of infection. Maximum depression of the CFU-S content and CFU-S per 10^6 bone marrow cells was observed on day 14, after the peak parasitemia had passed, and there was only a trace amount of the parasites detectable in the peripheral blood. The cellularity and the CFU-S content were at normal levels by day 23, about the time the infection was resolved (Fig 3).

In contrast to the bone marrow data, our assays showed an increase in CFU-S content in the blood and spleens of mice infected with Pca. Normal blood contained about 1.0 CFU-S/0.30 mL. On days 6, 14, and 23, the CFU-S in 0.30 mL was 15, >50 , and 2. The CFU-S content and cellularity of the spleen in uninfected mice contained 2,500 CFU-S and about 170×10^6 cells. On day 14, when the parasitemia was less than 1%, the CFU-S content reached 38,000, and the cellularity rose to 970×10^6 cells. By day 23 postinoculation, when the parasitemia was negative, the CFU-S content dropped to 15,000 and cellularity to 380×10^6 cells.

Table 1. Plasma Ep Titers in Mice Infected with *P. vinckei vinckei*, *P. berghei*, or *P. chabaudi adami*

Days Postinoculation	Parasitemia %	mU/mL	
		200 m	5h 6, 700 m
<i>P vinckei vinckei</i>			
2	±	7* (50)†	
3	10	3 (50)	
4	30	2 (50)	
5	70	700 (600)	1,100* (600)†
6	35	340 (660)	2,000 (1,100)
<i>P berghei</i>			
1	0	5 (50)	290 (300)
2	0	8 (50)	250 (80)
3	5	5 (50)	280 (120)
4	20	12 (75)	300 (160)
6	35	3,000 (3,400)	3,000 (2,400)
8	40	6,000 (4,800)	4,500 (4,200)
<i>P chabaudi adami</i>			
6	5	15 (50)	270 (100)
7	12	27 (50)	250 (180)
8	12	270 (110)	1,000 (400)
9	23	500 (130)	750 (280)
Uninfected	0	10 (50)	300 (270)

*Each value represents the mean of plasma samples that were pooled from 12 to 14 animals as determined by radioimmunoassay.

†Numbers in parentheses represent mean values of plasma samples determined by bioassay.

Table 2. The Six-Hour ^{59}Fe Uptake in Femoral Marrow and Spleen in Mice as a Function of Time After Inoculation with *P. vinckei vinckei* or *P. berghei**

Days Postinoculation	Parasitemia %	% ^{59}Fe 2 Femurs	% of Uninfected Femurs	Spleen Weight g	% ^{59}Fe Spleen	% ^{59}Fe per 0.10 g Spleen
Uninfected	0	2.4 $\pm$ 0.12	100 $\pm$ 7.0	0.11 $\pm$ 0.01	10.0 $\pm$ 0.63	9.2 $\pm$ 1.0
Phenylhydrazine	0	0.87 $\pm$ 0.06	36 $\pm$ 3.0	0.64 $\pm$ 0.05	27.0 $\pm$ 0.40	4.2 $\pm$ 0.30
<i>P. vinckei vinckei</i>						
1	0	3.1 $\pm$ 0.15	130 $\pm$ 8.7	0.11 $\pm$ 0.03	13 $\pm$ 0.58	12 $\pm$ 0.33
2	ND†	3.2 $\pm$ 0.14	130 $\pm$ 8.7	0.11 $\pm$ 0.05	11 $\pm$ 1.5	10 $\pm$ 0.47
3	ND	3.2 $\pm$ 0.14	130 $\pm$ 8.7	0.15 $\pm$ 0.03	14 $\pm$ 1.3	9.3 $\pm$ 2.1
4	2.3 $\pm$ 0.8	2.7 $\pm$ 0.27	110 $\pm$ 12.0	0.23 $\pm$ 0.05	17 $\pm$ 0.69	7.4 $\pm$ 1.6
5	14 $\pm$ 4	2.3 $\pm$ 0.06	94 $\pm$ 5.2	0.34 $\pm$ 0.05	11 $\pm$ 1.1	3.2 $\pm$ 0.57
6	55 $\pm$ 10	1.4 $\pm$ 0.13	58 $\pm$ 6.0	0.53 $\pm$ 0.13	12 $\pm$ 0.46	2.3 $\pm$ 0.56
7	85 $\pm$ 3	2.6 $\pm$ 0.09	110 $\pm$ 6.4	0.66 $\pm$ 0.22	13 $\pm$ 0.85	2.0 $\pm$ 0.67
<i>P. berghei</i>						
3	trace	2.9 $\pm$ 0.20	120 $\pm$ 0.10	0.14 $\pm$ 0.02	7.6 $\pm$ 2.3	5.5 $\pm$ 1.8
6	31 $\pm$ 4.5	1.2 $\pm$ 0.33	48 $\pm$ 14	0.29 $\pm$ 0.02	6.8 $\pm$ 2.4	2.3 $\pm$ 0.85
7	46 $\pm$ 4.5	0.80 $\pm$ 0.06	33 $\pm$ 2.9	0.54 $\pm$ 0.06	45 $\pm$ 1.8	8.3 $\pm$ 0.98
9	59 $\pm$ 4.2	0.82 $\pm$ 0.09	34 $\pm$ 4.0	0.51 $\pm$ 0.07	39 $\pm$ 3.2	7.6 $\pm$ 1.2
10	37 $\pm$ 2.9	0.65 $\pm$ 0.04	27 $\pm$ 2.1	0.77 $\pm$ 0.03	37 $\pm$ 1.0	4.8 $\pm$ 2.3
14	55 $\pm$ 2.4	0.95 $\pm$ 0.04	39 $\pm$ 2.5	0.61 $\pm$ 0.04	35 $\pm$ 3.0	5.8 $\pm$ 0.62

*All values are expressed as mean $\pm$ SE.

†ND = Not Done

Changes in the spleen. Splenomegaly has long been recognized as a characteristic response to malaria and other parasitic infections. The spleen in an infected animal increases seven to ten times over normal spleen weights (Table 4). Histologic examination of the spleen during the height of the response indicated that the cellular contents were predominantly granulocytic and lymphocytic with dispersed foci of erythroid elements. No histologic evidence of enhanced splenic erythropoiesis was apparent. The predominant granulocytic and lymphocytic character of the spleen was maintained until death occurred in the mice infected with Pb or Pvv. In mice infected with Pca the cellular character of the spleen began to alter after resolution of the

malaria, eventually returning to normal size and histologic appearances.

DISCUSSION

The data presented here together with the work of others^{13,14} demonstrate that ample amounts of functional erythropoietin are produced during infection with malaria. Indeed, during the later stages of infection with Pb, the EP plasma levels are not further increased by an hypoxic exposure, suggesting that the EP generating system is stimulated maximally (Table 1). Mice infected with Pvv and Pca are capable of responding to an additional hypoxic stimulus at or

Table 3. The Six-Hour Uptake of ^{59}Fe in the Femoral Marrow and Spleen in Mice as a Function of Time After Inoculation with *P. chabaudi adami**

Days Postinoculation	Parasitemia %	% ^{59}Fe 2 Femurs	% of Uninfected Femurs	Spleen Weight g	% ^{59}Fe Spleen	% ^{59}Fe per 0.10 Spleen
Uninfected	0	2.4 $\pm$ 0.12	100 $\pm$ 7.0	0.11 $\pm$ 0.01	10 $\pm$ 0.63	9.2 $\pm$ 1.0
<i>P. chabaudi adami</i>						
1	negative	2.8 $\pm$ 0.21	120 $\pm$ 11	0.08 $\pm$ 0.01	5.1 $\pm$ 0.64	6.3 $\pm$ 1.1
2	trace	2.8 $\pm$ 0.13	120 $\pm$ 7.8	0.07 $\pm$ 0.01	4.7 $\pm$ 1.3	6.8 $\pm$ 2.1
3	trace	3.1 $\pm$ 0.08	130 $\pm$ 7.1	0.08 $\pm$ 0.01	4.2 $\pm$ 0.39	5.2 $\pm$ 0.82
4	1.5 $\pm$ 0.22	1.9 $\pm$ 0.20	77 $\pm$ 9.0	0.18 $\pm$ 0.01	12.0 $\pm$ 1.2	6.6 $\pm$ 0.74
5	5.0 $\pm$ 0.68	1.5 $\pm$ 0.10	60 $\pm$ 5.0	0.27 $\pm$ 0.01	12.0 $\pm$ 0.56	4.6 $\pm$ 0.27
6	13.0 $\pm$ 1.6	0.87 $\pm$ 0.07	36 $\pm$ 3.4	0.44 $\pm$ 0.01	13.0 $\pm$ 1.5	2.9 $\pm$ 0.35
9	12.0 $\pm$ 1.0	0.64 $\pm$ 0.01	26 $\pm$ 1.4	1.0 $\pm$ 0.04	44.0 $\pm$ 0.96	4.3 $\pm$ 0.19
11	trace	0.34 $\pm$ 0.03	14 $\pm$ 1.4	1.3 $\pm$ 0.03	31.0 $\pm$ 1.7	2.3 $\pm$ 0.14
12	trace	0.42 $\pm$ 0.02	17 $\pm$ 1.2	1.1 $\pm$ 0.12	26.0 $\pm$ 1.9	2.4 $\pm$ 0.31
13	negative	0.30 $\pm$ 0.02	12 $\pm$ 1.0	1.1 $\pm$ 0.08	34.0 $\pm$ 0.78	3.1 $\pm$ 0.24
19	negative	0.64 $\pm$ 0.03	27 $\pm$ 1.8	0.77 $\pm$ 0.03	41.0 $\pm$ 1.3	5.3 $\pm$ 0.27
27	negative	2.1 $\pm$ 0.10	85 $\pm$ 5.8	0.31 $\pm$ 0.01	16.0 $\pm$ 0.9	5.0 $\pm$ 0.34

*Groups of six BALB/c female mice were injected IV with 0.50 μCi $^{59}\text{FeCl}_3$. All values are expressed as mean $\pm$ SE.

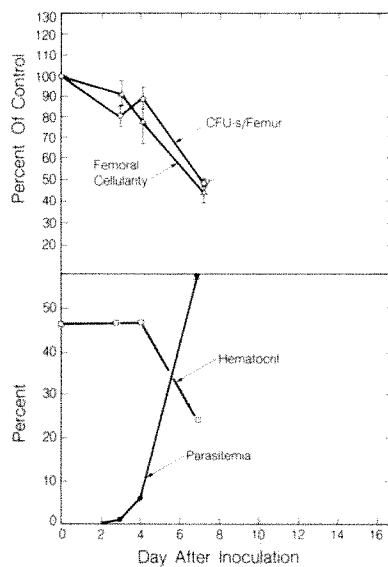


Fig 1. Changes in parasitemia, hematocrit, femoral cellularity, and CFU-S populations following IV inoculation of Balb/c female mice with 1×10^6 *Plasmodium vinckei vinckei*-infected red cells.

near the height of parasitemia. The reasons for the differences between Pvv, Pca, and Pb in the stimulation of EP production require further investigation. In any case, these results suggest that the lack of an adequate erythropoietic response during malaria infection is probably due to a failure of the erythroid progenitor cells to respond to EP, or a decreased production of erythroid progenitors, and/or a diminished production of multipotential stem cells.

The changes in bone marrow content of mice during the course of infection with malaria show striking differences between the three species of parasites used in this study.

Infection with the highly virulent murine malaria, Pvv, led

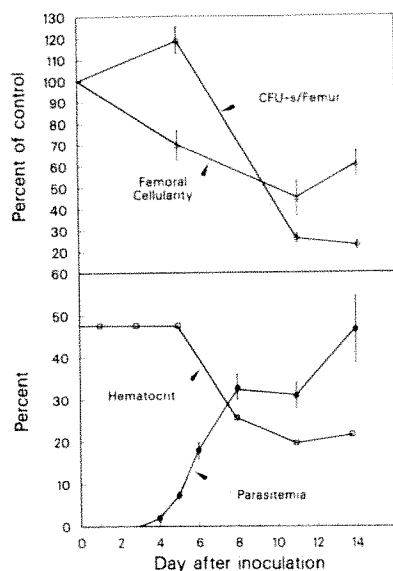


Fig 2. Changes in parasitemia, hematocrit, femoral cellularity, and CFU-S populations following IV inoculation of Balb/c female mice with 1×10^6 *Plasmodium berghei*-infected red cells.

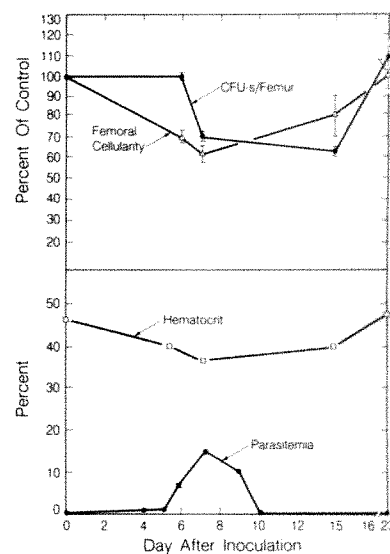


Fig 3. Changes in parasitemia, hematocrit, femoral cellularity, and CFU-S populations following IV inoculation of Balb/c female mice with 1×10^6 *Plasmodium chabaudi adami*-infected red cells.

to high parasitemias, low hematocrits, and death in seven to nine days (Fig 1). Associated with these events is a rapidly declining total femoral cellularity dropping to less than 50% of normal at the time of death. Similarly, the stem cell content showed a parallel decline, dropping to 45% of the normal level before death of the mice.

The temporal pattern of cellular changes in the bone marrow of mice infected with Pb differed from both Pvv and Pca infections. Pb stimulated stem cell populations during the early phase of infection even though total bone marrow cellularity was declining. It is possible that the stimulation of bone marrow stem cells by Pb is related to its propensity for immature erythrocytes and its predilection for recycling in the bone marrow.¹⁹

Pb has a less rapid increase in parasitemia and reaches lower levels of parasitism than does Pvv. On the other hand, Pb eventually kills its host shortly after stem cell populations drop to less than 25% of normal.

A substantial migration of CFU-S from bone marrow to spleen occurs during the early phase of the infection. However, it is apparent from histologic and functional observations that the spleen from an infected animal, enriched with stem cells, does not meet the erythropoietic and hemopoietic needs of the host. The data on six hours uptake of ^{59}Fe by bone marrow and spleen tissue during the course of infection with the three species of plasmodia indicated that the depressed erythropoietic capacity of marrow is paralleled by a similar depression in the spleen. The evidence for decreased erythropoietic capacity in both sites is apparent irrespective of the seven- to tenfold increase in spleen cellularity that is associated with malaria infection. One must conclude that at least during the first days of infection the enlarged spleen does not compensate for the lost erythropoietic capacity of the bone marrow or, probably, other depressed hemopoietic functions. It has been recently reported that the depressed

Table 4. Changes in Female Balb/c Mice After Inoculation with 1×10^5 Red Blood Cells Infected with Murine Malaria

Donor N	Days Post-inoculation	Parasitemia %*	Cells $\times 10^6$ /Femur	CFU-S/ 10^6 BMC	CFU-S/Femur*	Hematocrit %*	Body Weight g*	Spleen Weight g*
Uninfected controls								
16	—	—	13.7 ± 0.50	285 ± 7.0	$390C \pm 86$	47 ± 0.04	20.7 ± 0.19	0.10 ± 0.01
<i>P. vinckei vinckei</i>								
5	3	<1%	12.4 ± 0.46	250 ± 12	$310C \pm 145$	47 ± 0.04	18.5 ± 0.39	0.11 ± 0.01
4	4	5.2 ± 1.2	10.5 ± 1.2	324 ± 18	$340C \pm 145$	47 ± 0.04	19.3 ± 0.35	0.30 ± 0.02
5	7	65 ± 3.3	6.1 ± 0.57	312 ± 10	$190C \pm 94$	24 ± 0.92	18.5 ± 0.21	0.64 ± 0.02
<i>P. berghei</i>								
4	5	8.3 ± 2.6	9.9 ± 0.76	473 ± 20	$470C \pm 190$	48 ± 0.65	20.2 ± 0.55	0.25 ± 0.01
4	11	32 ± 3.1	6.4 ± 0.99	149 ± 7.0	$100C \pm 73$	20 ± 3.0	15.8 ± 0.92	0.74 ± 0.12
5	14	55 ± 5.6	8.6 ± 0.63	105 ± 7.0	$90C \pm 63$	24 ± 1.0	13.8 ± 0.23	0.65 ± 0.08
<i>P. chabaudi adami</i>								
4	6	5.5 ± 0.98	9.4 ± 0.26	420 ± 21	$400C \pm 194$	40 ± 1.4	20.0 ± 0.48	0.41 ± 0.01
4	7	15.3 ± 1.1	8.2 ± 0.75	340 ± 13	$280C \pm 93$	36 ± 2.5	21.5 ± 0.29	0.68 ± 0.12
4	14	<1%	11.1 ± 0.93	220 ± 11	$240C \pm 155$	39 ± 1.9	22.0 ± 0.91	1.10 ± 0.03
4	23	zero	13.8 ± 0.70	315 ± 21	$440C \pm 270$	48 ± 0.48	22.5 ± 0.50	0.34 ± 0.01

*Mean $\pm$ SE.

BMC = Bone marrow cells.

immunologic response in patients during infection and convalescence was associated with a loss of T lymphocytes—both helper and suppressor T cell subsets—from the peripheral blood.²⁰ These findings of a loss of peripheral lymphocytes may result from stem cell depletion and may represent a fundamental contributor to the pathology of malaria.

Early studies on the mechanism of stem cell depletion suggest that a humoral substance is involved. A possible mechanism of action might be due to the effects of tumor necrosis factor (TNF), which is produced by macrophages during the processing of malaria antigen. It has been reported that TNF inhibits adipocyte gene expression.^{21,22} Adipocytes are a major cellular component of the adherent stromal bone marrow and provide an essential environment for renewal and differentiation of the nonadherent hemopoietic stem cells. The inhibitory action of malaria-induced TNF might account for both the drop in total cellularity and the depletion of hemopoietic stem cells.

The effect of a depleted hemopoietic stem cell population is of importance to the outcome of malaria infection. These multipotential stem cells are known to be the source of all blood cells, and their loss might reduce the host's capacity to meet physiologic needs during stress of the disease.

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Prevention of Platelet Alloimmunization in Dogs With Systemic Cyclosporine and by UV-Irradiation or Cyclosporine-Loading of Donor Platelets

By Sherrill J. Slichter, H. Joachim Deeg, and Michael S. Kennedy

To study the immunosuppressive effects of three different treatments, 30 dogs received at weekly intervals eight platelet transfusions from a single random donor dog. The three experimental protocols were (1) daily oral cyclosporine (Cs) treatment of recipients; (2) in vitro ultraviolet (UV)-irradiation of donor platelets; and (3) Cs-loading of donor platelets. All nine recipients of Cs, 11/12 (92%) recipients of UV-irradiated platelets, and 5/9 (56%) recipients of Cs-loaded donor platelets remained nonimmunized to repeated transfusions of donor platelets. In contrast, only 3 of 21 untreated controls (14%) were not alloimmunized by donor platelets. Moreover, 44% to 67% of the nonimmunized recipients remained tolerant to continued

platelet transfusions from their original donor even after experimental therapy was discontinued. Forty-three percent to 100% of transfusions from secondary donors were also accepted without causing alloimmunization, suggesting that tolerance induced by prior treatment was not specific for the primary donor. However, survival of both the original and secondary donor platelets was reduced to about half the starting level, suggesting that some immune response to platelets had occurred. Also, recipients immunized by their original donor's platelets frequently developed refractoriness to platelets from other donors.

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TO PREVENT or modify the immune response to transplanted tissue, a number of immunosuppressive treatments have been developed, such as irradiation, chemotherapeutic regimens, polyclonal or monoclonal antibodies, and the use of cyclosporine (Cs).¹

A more recent approach is the modification of donor cells or tissues. Certain antigen-presenting cells such as macrophages or dendritic cells are now known to be necessary for the donor's tissue to be recognized by the recipient. Thus, several attempts have been made to eliminate or inactivate these cells.²⁻⁴ Reportedly, successful inactivation has been achieved by maintaining long-term cell or tissue cultures in a high concentration of oxygen,⁵ by using monoclonal antibodies (MoAbs) directed at Class II antigens (expressed on antigen-presenting cells),⁶ and by exposing donor cells or tissue to ultraviolet (UV) light in the intermediate wavelength spectrum.^{3,7,8}

Although there is substantial information on the value of these and other agents in organ transplantation, little is known about their ability to prevent immune recognition of repeatedly transfused cells. In particular, we are concerned about alloimmunization to transfused platelets, since it represents a major problem in the management of thrombocytopenic patients. Studies suggest that 40% to 100% of repeatedly transfused patients become alloimmunized, often after only a few transfusions.^{9,10} We have previously used a dog platelet transfusion model to evaluate methods of pre-

venting platelet alloimmunization¹¹ (and unpublished data). This report describes further studies using this model to evaluate recipient treatment with Cs and UV-exposure or Cs-loading of donor platelets as methods of preventing platelet alloimmunization.

METHODS

Selection of Donor and Recipient Dogs

The dogs used for these experiments were of various breeds and of either sex. Dogs were vaccinated (DHL and parvo virus) and quarantined for one to two months to exclude any diseases. In addition, two or three autologous ⁵¹Cr-labeled platelet survival measurements (see below) were performed on each recipient dog to ensure baseline normal platelet kinetics before donor transfusions were initiated.

During the course of the study, eight different dogs were used as donors for the 30 platelet transfusion recipients. All of the donors and most of the recipients were typed for serologically determined dog histocompatibility antigens (DLA-A and B).¹² In all instances tested, donors and recipients were incompatible for one or more DLA-antigens. In addition, for some studies mixed lymphocyte cultures were performed with cells obtained from donors and from recipients both pre- and posttransfusion.¹³ In all tested donor-recipient pairs, cells showed reciprocal stimulation both before and after the donor transfusions and experimental therapy.

Survival of Radiochromium(⁵¹Cr)-Labeled Platelets

Platelets were prepared from the donor dog and labeled with radiochromium as previously described.¹⁴ Every week 50 mL of blood was drawn from each donor for each recipient into 7.5 mL of acid-citrate-dextrose (ACD). A platelet concentrate was prepared by differential centrifugation and labeled with 300 μ Ci of ⁵¹Cr. After infusion of these platelets, blood samples (3 mL blood drawn into 0.1 mL potassium EDTA) were obtained at one hour, twice on the second day, and once daily during the third and fourth days. Survival was calculated by least squares computerized-fitting of the radioactivity in the blood samples.^{15,16} Autologous platelet survival in 56 normal dogs averaged 5.1 ± 0.9 days (mean $\pm$ 1 SD).

Platelet recovery was calculated as follows by extending the survival curve to time zero (T0):

$$\text{Recovery (\%)} = \frac{\text{Radioactivity at T0} \times \text{blood vol (estimated at 80 mL/kg)}}{\text{Total radioactivity of injected platelets}} \times 100$$

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Autologous recovery in normal dogs was $51\% \pm 12\%$ (mean ± 1 SD) of the injected platelets.

Alloimmunization was defined as a decrease in the recovery of labeled donor platelets to $<5\%$ at 24 hours posttransfusion. One additional donor transfusion was given after the development of alloimmunization to confirm the accuracy of the end point. Thus, two serial donor platelet transfusions showing poor survivals were required to categorize a recipient as alloimmunized.

Alloimmunization of Untreated Dogs

Control values for induction of alloimmunization were determined by transfusing 21 recipient dogs with platelets from one of 11 donors. Twenty of 21 (95%) became immunized after an average of 3.1 ± 0.7 (range one to 12) transfusions¹¹ (Fig 1). As 18/21 (86%) of these recipient controls were immunized by eight or fewer transfusions, an eight-week transfusion schedule was selected for the subsequent alloimmunization studies, ie, the treated dogs received platelets from their donor until alloimmunization developed or a maximum of eight transfusions had been given.

Treatment Programs

Systemic cyclosporine. Cyclosporine (Cs, Sandoz, Basel, Switzerland) was emulsified in corn oil¹⁷ and administered orally in doses of 15 mg/kg/d. Treatment was started on the day of the first platelet transfusion and continued for up to eight weeks or until alloimmunization occurred. To document absorption of drug and maintenance of adequate levels, Cs serum levels were monitored in two recipient dogs throughout their treatment using a radioimmunoassay (RIA), as previously described.¹⁸ Serum drug levels were obtained every three to five days as trough levels just before the next daily dose. For one dog the average trough levels were 105 ± 68 (range 22 to 255) ng/mL and for the second dog 139 ± 30 (range 108 to 192) ng/mL. Therapeutic Cs drug levels are considered to range between 100 to 400 ng/mL.

UV-exposed platelets. After radiochromium labeling, platelets were resuspended in a volume of 6 to 10 mL of Ringer's-citrate-dextrose (RCD) and placed (to a depth of 1.5 mm) in an open Petri dish and continuously agitated on a shaker platform during UV exposure. UV irradiation was delivered by a mineral light lamp (UVP, San Gabriel, CA) providing wavelengths between 220 to 290 nm. The dose delivered was determined by using a Black-Ray Ultra-violet Meter (Model J225, UVP) with a maximum sensitivity at 254 nm. After UV exposure the contents of the Petri dish were aspirated into a syringe for injection.

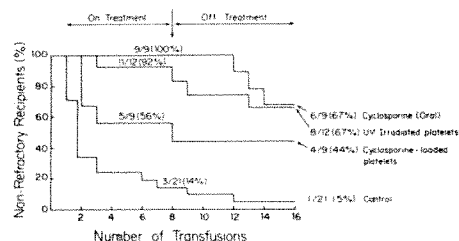


Fig 1. Prevention of platelet alloimmunization. Shown are the percentages of recipients who did not become immunized by eight platelet transfusions from a single unrelated donor while on treatment followed by eight transfusions from the same donor while off therapy. Data in treated dogs are compared to results in untreated control dogs. At the end of each transfusion sequence, the number of dogs not immunized to donor platelets is given as a fraction of the number of dogs studied. The treatment programs were (1) oral Cs given to the recipient; (2) UV irradiation of platelets; and (3) Cs loading of platelets.

Cyclosporine-loaded platelets. Radiochromium-labeled donor platelets, resuspended in 4.6 mL of RCD, were incubated for 1 hour at 22 °C with 55 µg/mL of Cs prepared by adding 0.25 mg (5 µL of a 50 mg/mL solution) of Cs in ethanol: Cremaphor (intravenous [IV] solution from Sandoz) to the platelets. Following incubation the platelets were injected IV, the recipients receiving only 0.25 mg of Cs per injection. Blood taken immediately and one hour after injection contained no measurable Cs.

Uptake of Cs was assessed by measuring the platelet Cs content by high-pressure liquid chromatography and by using radiolabeled ³H-cyclosporine provided by Dr Eric Wiskott of Sandoz. The amount of Cs incorporated was 6% to 12% of that added. There was no difference in Cs uptake in donor platelets between dogs who subsequently became immunized and those who did not.

Further Transfusion Studies

Any recipient not immunized by eight transfusions while receiving the experimental treatment was given an additional eight transfusions from the same donor while off treatment or until immunization developed, whichever occurred first. When the transfusion schedule from the original donor was completed, the recipient dogs then received transfusions from one to three additional donors (referred to as secondary donors) while off experimental treatment. Transfusions were provided from the randomly selected secondary DLA-incompatible donors until immunization developed or until a maximum of eight transfusions were given.

Statistical Analysis

Mean values of experimental and control groups were compared by Student's *t* test.¹⁹

RESULTS

Autologous Platelet Transfusions

Recovery and survival measurements with experimental treatment. To ensure that treatment did not adversely affect the viability of transfused platelets, autologous platelet recovery and survival measurements were performed for each experimental treatment. Four dogs were given oral Cs during the transfusion of radiolabeled autologous platelets. Recoveries and survivals with this treatment were not significantly different from those in 56 untreated controls ($P > 0.10$) (Table 1).

Doses of UV irradiation given to autologous platelets were 200, 400, or 600 µW/cm² for one minute (total doses of 12, 24, and 36 mJ/cm², respectively). These doses did not affect autologous platelet survival (Table 1). However, in two dogs

Table 1. Recovery and Survival of ⁵¹Cr-Labeled Autologous Platelets

Treatment	Number of Dogs Tested	Platelet	
		Recovery (%)*	Survival (Days)*
None	56	51 $\pm$ 12	5.1 $\pm$ 0.9
Systemic Cs	4	58 $\pm$ 9	4.1 $\pm$ 0.6
UV-exposed platelets	14	52 $\pm$ 6	4.3 $\pm$ 1.1
Cs-loaded platelets	5	57 $\pm$ 6	4.9 $\pm$ 1.3

*Given as the mean ± 1 SD.

By Student's *t* test, none of the treatment programs produced platelet recovery and survival measurements that were statistically different from untreated controls.

that received $600 \mu\text{W}/\text{cm}^2$ for ten minutes (total dose of $360 \text{ mJ}/\text{cm}^2$), there was no recovery or survival of autologous platelets (data not shown). Cyclosporine-loading in five dogs did not reduce platelet viability (Table 1).

Allogeneic Platelet Transfusions

Recipient treatment with oral cyclosporine. Of the nine recipients who received oral Cs therapy, none became immunized after eight transfusions from a single random donor (Fig 1). These results were significantly different from those in the 21 untreated control dogs ($P < 0.001$); only three of them (14%) remained unimmunized after eight transfusions. Furthermore, 6/9 recipients (67%) were still responsive to transfused platelets when Cs was stopped, even after eight additional transfusions from the same donor ($P < 0.001$, compared to controls). The other three recipients became immunized after four, five, and six additional donor transfusions given off treatment (Fig 1).

Although zero time-recovery values for the original donor's platelets in the nonimmunized recipients were within the normal range throughout the 16 transfusions (eight on and eight off treatment), donor platelet survivals were only 2.2 ± 0.5 days, ie, about half normal (Fig 2).

UV exposure of donor platelets. Overall, 11/12 recipients (92%) of UV-exposed donor platelets (12 to $36 \text{ mJ}/\text{cm}^2$) were protected from alloimmunization. These UV-exposed donor platelets produced a significantly lower rate of alloimmunization than unmodified platelets given to control recipients ($P < 0.001$) (Fig 1).

To determine if an effective dose of UV irradiation could be achieved through a blood bag, platelets from two donors were individually irradiated through a piece of plastic cut from a Fenwall platelet storage bag (PL 732, Fenwall Laboratories, Deerfield, IL) that was laid over the Petri dish containing the platelets. (As the UV penetrance through the plastic bag was poor with the available UV source, a maximum dose of $100 \mu\text{W}/\text{cm}^2$ could be obtained.) However, by irradiating at $100 \mu\text{W}/\text{cm}^2$ for four minutes, the equivalent

of our standard dose of $400 \mu\text{W}/\text{cm}^2$ for one minute was achieved, ie, the total UV dose given to platelets by each of these exposures was $24 \text{ mJ}/\text{cm}^2$. Two recipients received donor platelets irradiated through the plastic and, as controls, two others received the same donor's platelets given the exact exposure ($100 \mu\text{W}/\text{cm}^2$ for four minutes) without interposition of plastic; all four recipients were protected from alloimmunization (data included above).

When transfusions from the original platelet donor were continued off treatment, three recipients became immunized after one, two, and five transfusions, respectively (Fig 1). Thus, 8/12 recipients (67%) were not immunized by a total of 16 transfusions, eight on and eight off treatment ($P < 0.001$ compared to controls).

The eight nonimmunized recipients were given platelets from two or three other donors until immunization occurred or a maximum of eight transfusions from each of these other donors had been given. Prior treatment prevented alloimmunization to platelets from 10/23 (43%) of the secondary donors tested (Table 2). However, compatible donors were found for only four (50%) of the nonimmunized recipients. After completion of these secondary donor transfusion studies, six of the nonimmunized recipients were transfused with platelets from their original donor and two (33%) were immunized.

Three of the four immunized recipients were each given platelets from one or two other donors; only 1/5 of the secondary donors' platelets (20%) remained compatible during eight transfusions (Table 2).

Recovery of UV-exposed donor platelets in the nonimmunized recipients was normal, but survival was reduced (Fig 3). However, the survival of donor platelets off treatment (3.1 ± 0.4 days) was somewhat better than on treatment (2.5 ± 0.3 days). Recoveries from the secondary donors in the nonimmunized recipients averaged $42\% \pm 10\%$, and survivals were 2.9 ± 1.3 days.

Cyclosporine-loaded donor platelets. Undesirable side effects of systemic Cs are sometimes observed in immunosuppressed patients. We therefore evaluated the use of in vitro Cs-loaded donor platelets for preventing alloimmunization. Five of 9 (56%) of the recipients of Cs-loaded platelets did not become alloimmunized (Fig 1) ($P < 0.05$ compared to untreated controls). Furthermore, 4/9 or 44% remained nonimmunized to the original donor's platelets even off treatment ($P < 0.001$). In addition, none of the three nonimmunized recipients tested were alloimmunized after receiving untreated platelets from either of two secondary donors (Table 2).

In contrast to the data in the nonimmunized recipients, all three tested recipients who had become immunized to their original donors' platelets also were alloimmunized after receiving fewer than eight transfusions of unmodified platelets from five secondary donors (Table 2).

Recoveries of the original donor's platelets in the recipients were within the normal range for both the treated and untreated transfusions while survivals were reduced (Fig 4). Recovery of the secondary donor's platelets averaged $51\% \pm 14\%$ in the nonimmunized recipients and survival was 3.0 ± 1.0 days.

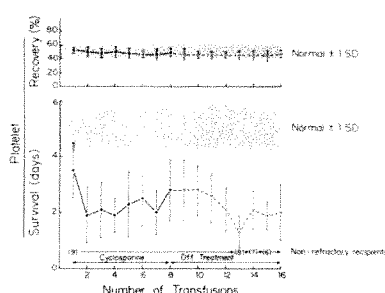


Fig 2. Viability of platelets in nonimmunized Cs-treated recipients. The upper part of the figure shows the mean recovery (± 1 SD) of donor platelets in the recipient on treatment (closed circles) and off treatment (open circles). The normal range (± 1 SD) for dog platelet recovery in 56 normal animals is shown as the hatched area. The lower panel shows the mean survival (± 1 SD) of donor platelets in recipients on treatment (closed circles) and off treatment (open circles). The number of recipient animals who were not immunized by each transfusion is shown below each survival. The range of normal platelet survival (± 1 SD) in dogs is shown as the hatched area.

Table 2. Response to Platelets From Secondary Donors

Treatment Given to Original Donor's Platelets	Refractory Recipients*		Nonrefractory Recipients*	
	Number of Recipients Tested	No. Nonrefractory Secondary Donors/No. Tested (%)†	Number of Recipients Tested	No. Nonrefractory Secondary Donors/No. Tested (%)†
UV-exposed platelets	3	1/5 (20%)	8	10/23 (43%)
Cs-loaded platelets	3	0/5 (0%)	3	6/6 (100%)

*In each treatment group, recipients refractory as well as nonrefractory to their original donor's platelets were transfused with platelets from other donors, referred to as secondary donors, while off treatment (see text).

†The number of secondary donors to whom the recipients did not become refractory with eight transfusions is given compared to the number of secondary donors transfused.

DISCUSSION

Specific attempts to prevent platelet alloimmunization in chronically transfused thrombocytopenic patients, other than by using single donor platelets^{20,21} or leukocyte-free blood products,^{22,23} have not been reported. Our studies have shown that both immunosuppression with systemic Cs and UV irradiation of donor platelets were almost uniformly successful (100% and 92%, respectively) in preventing alloimmunization to eight weekly platelet transfusions from a single random donor. These results are much better than the nonimmunized rate of only 14% in untreated controls ($P < 0.001$ for both treatments). Furthermore, almost two thirds of the nonimmunized previously treated recipients remained unimmunized after eight additional transfusions from the same donor, suggesting the induction of a tolerant state. To determine if this tolerance was specific for the original donor's platelets, platelets from other random donors were transfused without treatment of the recipient or the donor's platelets. The number of Cs-treated recipients evaluated for such specificity was too small to permit a firm conclusion (data not given). However, among the recipients tolerant to eight UV-treated and eight nontreated platelet transfusions from their original donor, platelets from 43% of the secondary donors tested were also compatible. In addition, there was some evidence that maintenance of this quasitolerant state required continuous transfusions of the

presumed tolerizing antigen; interruption of the transfusion program for a period of months resulted in immunization by untreated transfusions from the primary donor in at least one third of the recipients tested. Similar observations have been made in other models of tolerance.²⁴

We were concerned about the substantial immunosuppressive effects of Cs on human subjects as well as its toxicity to liver and kidneys.²⁵ Therefore, in analogy to the use of vinblastine-loaded platelets in patients with autoimmune thrombocytopenic purpura,²⁶ we evaluated the ability of Cs-loaded donor platelets to prevent immunization. Although 56% of the recipients were apparently unable to recognize Cs-loaded donor platelets, this treatment was inferior to that obtained with either systemic Cs or UV-irradiated donor platelets ($P < 0.001$). However, treatment with Cs-loaded donor platelets was very successful in inducing a state of tolerance not only to untreated platelets of the original donor but also to secondary donors.

In summary, our studies show that some treatment programs that prevent the rejection of organ allografts were successful in reducing the recognition of repeatedly transfused donor platelets in a canine transfusion model. Furthermore, to some extent, these treatments induced a state of "nonspecific tolerance" that persisted even after discontinuation of experimental therapy. However, maintenance of the tolerant state apparently required continuous exposure to the tolerizing antigen. Conversely, in those recipients who

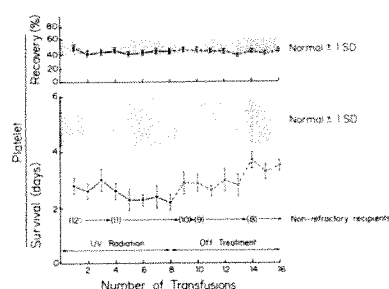


Fig 3. Viability of UV-exposed platelets in nonimmunized recipients. The upper part of the figure shows the mean recovery (± 1 SD) of donor platelets. Transfusions given after UV exposure (on treatment) are shown as closed circles and normal transfusions (off treatment) as open circles. The normal range (± 1 SD) for dog platelet recovery in 56 normal animals is shown as the hatched area. The lower panel shows the mean survival (± 1 SD) of donor platelets after UV exposure (closed circles) and without UV exposure (off treatment, open circles). The number of recipient animals who were not immunized by each transfusion is shown below each survival. The range of normal platelet survival (± 1 SD) in dogs is shown as the hatched area.

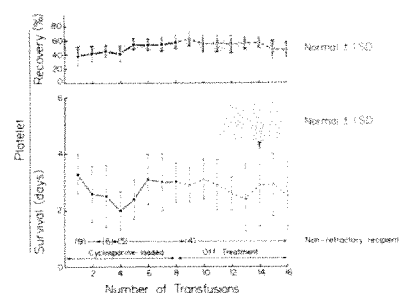


Fig 4. Viability of Cs-loaded platelets in nonimmunized recipients. The upper part of the figure shows the mean recovery (± 1 SD) of donor platelets. Transfusions with Cs-loaded donor platelets are shown as closed circles and with normal platelets (off treatment) as open circles. The normal range for dog platelet recovery in 56 normal animals is shown as the hatched area. The lower panel shows the mean survival of Cs-loaded donor platelets (closed circles) and off treatment (open circles). The number of recipient animals who were not immunized by each transfusion is shown below each survival. The range of normal platelet survival in dogs is shown as the hatched area.

became immunized by the original donor's platelets either on or off therapy, alloimmunization usually also developed in response to untreated platelets from other donors.

We also found that the survival of allogeneic donor platelets in every treatment program was consistently reduced as compared to similarly treated autologous platelets. Therefore, we conclude that some degree of alloimmunization to donor platelets probably occurred in recipients who were by definition considered to be nonimmunized. However, as autologous platelet survivals on treatment were only performed on one occasion, we cannot exclude the possibility that there was some cumulative effect of the treatment on donor platelet survival measurements. The recovery of platelets from both the original and secondary donors in the nonimmunized recipients was not compromised, and platelet survival remained about half normal; therefore, useful transfusion support was achieved by this approach. Furthermore, previous studies using *in vitro* platelet aggregation tests, have shown that human platelets maintain normal function after UV exposure²⁷ or Cs loading.²⁸ Although the applicability of our results to man has not been tested, we believe these studies provide a sufficient basis to consider human experiments.

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Expression of Two Natural Killer Cell Antigens, H-25 and H-366, by Human Immature Myeloid Cells and by Erythroid and Granulocytic/Monocytic Colony-Forming Units

By David Wisniewski, Robert Knowles, Maria Wachter, Annabel Strife, and Bayard Clarkson

Two monoclonal antibodies (MoAbs), H-25 and H-366, shown previously to react with human peripheral blood large granular lymphocytes with natural killer (NK) cell activity and some peripheral blood monocytes, have now been shown to also react with a significant proportion of the myeloid and erythroid precursor cells in human bone marrow and peripheral blood. In FACS IV cell sorting and immune rosetting of bone marrow cells, the antigens recognized by H-25 and H-366 were found to be expressed on most blasts and promyelocytes but sequentially fewer of the more mature cells of the myeloid lineage. Both antigens were also found on most monocytes but only a minor proportion of lymphoid and nucleated red cells in the bone marrow. In vitro assays detecting hematopoietic colony-forming units revealed that these antigens are expressed by virtually all mature erythroid colony-forming units (day-7 CFU-E), and the majority of the more primitive

erythroid burst forming units (day-14 BFU-E). H-25 but not H-366 was also found on a variable proportion of the day-7 and day-14 granulocytic/monocytic colony-forming units (CFU-GM) in the bone marrow. The same type of precursor cells are also found in the H-25 and H-366 positive cell populations isolated from peripheral blood. In preliminary testing of cells from acute leukemic patients, FACS analysis showed that both antigens are also expressed on leukemic cells from patients with T cell acute lymphocytic leukemia and with myeloid leukemias. These studies demonstrate that the H-25 and H-366 positive NK cells in the peripheral blood retain some of the cell surface properties of early hematopoietic precursor cells, thus providing further evidence supporting the bone marrow origin of NK cells.

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IN VITRO semisolid culture techniques have facilitated numerous studies on the regulation of hematopoiesis at the progenitor cell level. While it is clear from these studies¹⁻³ that proliferation and differentiation of hematopoietic progenitor cells are mediated by accessory cell factors and humoral factors, further understanding of the molecular mechanisms involved requires the purification of progenitor cells as well as the regulatory factors acting on them.

Recently our laboratory has been pursuing the purification of hematopoietic progenitor cells and has described a negative selection method for substantially enriching CFU-GM and BFU-E from normal human peripheral blood.^{4,5} Using this method, B lymphocytes, monocytes, and certain high affinity Fc receptor-bearing cells are depleted from mononuclear cells by immunoadherence to plastic plates coated with rabbit antihuman IgG. Cells not attached to these plates are subsequently depleted of T lymphocytes by coating with the pan-T murine monoclonal antibody LyT-3 before their removal by immunoadherence to plastic plates coated with goat antimouse IgG. The final "null" cell fraction is highly enriched for CFU-GM and BFU-E.

More recently, in attempts to further purify these progenitor cells, we sought to remove contaminating natural killer (NK) cells (non-T, non-B cells that display spontaneous cytotoxic activity against a variety of normal, neoplastic, or virus-infected cells⁶) from the null cell fraction using two previously described NK cell reactive monoclonal antibodies.⁷ Both of these antibodies, designated H-25 and H-366, have previously been shown to react with peripheral blood NK cells and monocytes but do not react with Leu-1 positive T lymphocytes, B lymphocytes, granulocytes, red blood cells, or platelets.

Preliminary immunoadherence experiments with the null cell fraction after reaction with these monoclonal antibodies (MoAbs) suggested, however, that antibody H-25 was cross-reactive with CFU-GM and BFU-E, while antibody H-366 was crossreactive with BFU-E. These preliminary observations prompted us to examine in more detail the expression of

antigens detected by H-25 and H-366 by hematopoietic progenitor cells from both normal bone marrow and peripheral blood. Thus, H-25 and H-366 antigen positive (+) and negative (-) cell fractions were obtained by FACS IV sorting and immune rosetting methods. Cell fractions were examined morphologically and also assayed for erythroid and granulocytic/monocytic colony-forming units using well established in vitro semisolid culture techniques. In addition, cells from some fresh acute leukemic samples as well as some established human cell lines were examined for H-25 and H-366 antigen expression by FACS. The results of these studies are the subject of the present report.

MATERIALS AND METHODS

Monoclonal antibodies. Production and characterization of monoclonal antibodies (MoAbs) H-25 and H-366 have been previously described in detail.⁷ Briefly, spleen cells from Balb/c mice immunized with the T cell acute lymphocytic cell line HSB-2 were fused with the P3 NS-1/1-ag 4-1 myeloma cell line by the method of Kohler and Milstein. Two hybrid clones (H-25 and H-366) were found to produce antibodies that bound to the parent HSB-2 cells

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but not to human B cell lines. Both hybrids were subsequently passaged as ascites tumors in pristane primed Balb/c mice.

Further characterization of both antibodies revealed similar tissue distribution. Thus, H-25 and H-366 were found in indirect immunofluorescence assays to react with a subset of peripheral blood mononuclear cells (MNC) that have the morphological characteristics of large granular lymphocytes (LGLs) and, furthermore, that contain most of the NK and K cell activity. Both antibodies were also found to react with a variable number of peripheral blood monocytes, a significant proportion of thymocytes (obtained from infant thymuses), but neither antibody was reactive with Leu-1 + T lymphocytes, B lymphocytes, granulocytes, red blood cells, or platelets. In a radioimmune trace-binding assay, H-25 and H-366 were found to react with all T cell lines tested and with the monocytic cell line U937 but weakly or not at all with cell lines of B, myeloid, or erythroid origin. Antibodies H-25 and H-366, which are IgG₁ and IgG_{2b} subclass specific, respectively, immunoprecipitate two polypeptide chains of apparent molecular weights of 96 and 53 kilodaltons from surface labeled HSB-2 cells, H-25 bringing down the former band and H-366 the latter.

In some of the experiments described below, MoAbs MO-1, MY-4, MY-8, N901, B-1 (kindly supplied as ascitic fluids by Dr James Griffin, Harvard Medical School, Cambridge, MA), Leu-1, Leu-4 (kindly provided as culture supernatants by Dr Robert Evans, Sloan-Kettering Institute, New York), and Leu-7 (Becton Dickinson, Mountain View, CA) were used. The production and characterization of these MoAbs have been previously described in detail. Anti-MO-1, which recognizes an antigenic determinant associated with the CR3 receptor, is found on monocytes, granulocytes, and approximately 80% of NK cells.⁸ B-1 identifies a 35,000 molecular weight antigen found specifically on cells of B lineage.⁹ MY-4 and MY-8 antigens are expressed by monocytes, and MY-8 is additionally expressed by cells of the granulocytic series.¹⁰ Leu-1 and Leu-4 are pan-T cell antigens.^{11,12} Anti-N901 detects an antigen, present at low density, on all NK cells and no other subpopulation of peripheral blood cells.¹³ Anti-Leu-7 is an LGL specific antibody that reacts with approximately 50% to 90% of NK cells.¹⁴

Source of cells. Heparinized, venous human peripheral blood (50 to 100 mL) was obtained from healthy volunteers. Human posterior iliac-crest bone marrow was obtained from normal volunteers by aspiration into preservative-free, heparin-containing syringes. Peripheral blood (10 to 20 mL) was additionally collected from these donors as a source of autologous serum. Written, informed consent was always given by the donors.

Human acute leukemia cells. Cells were obtained from the bone marrow and peripheral blood of seven patients with acute myelogenous leukemia (AML), two patients with T cell acute lymphocytic leukemia (T-ALL), and two patients with pre-B Calla⁺ (common acute lymphoblastic leukemia antigen) acute lymphoblastic leukemia (pre-B, ALL). Standard clinical, morphological, and cytochemical criteria were used to establish the diagnosis in each case, and the diagnosis was confirmed by surface marker analysis using a panel of MoAbs. AML patients were subclassified according to the FAB (French-American-British) classification system as AML (M2), acute myeloblastic leukemia with maturation (three patients); APL (M3), acute promyelocytic leukemia (two patients); AMOL (M5A), acute monocytic leukemia without maturation (one patient); and AMOL (M5B), acute monocytic leukemia with maturation (one patient).

Cell lines. Established human cell lines were grown in RPMI 1640 (GIBCO, Grand Island, NY) containing 15% fetal calf serum (FCS) (Hy-Clone, Logan, UT) and 1% penicillin-streptomycin-neomycin (PSN). Their origins are listed in Table 1.

Cell preparation from normal bone marrow and peripheral blood. Normal bone marrow cells were harvested after centrifuga-

tion of the aspirates at 1,000 RPM for ten minutes and suspended in McCoy's 5A modified medium (GIBCO) containing 10% fetal calf serum (FCS) (hereafter referred to as complete medium). Platelets were removed from the cell suspension by centrifugation on a Percoll (Pharmacia, Piscataway, NJ) density solution ($d = 1.050\text{g/mL}$) for 20 minutes at 2,000 RPM. At this density platelets remain at the interface while all nucleated cells are pelleted. After washing the pelleted cells once and resuspending in complete medium, light-density bone marrow cells were obtained by centrifugation over Ficoll-Hypaque (Pharmacia, $d = 1.077\text{g/mL}$). Light-density cells, harvested at the interface, were washed twice, resuspended in complete medium, and incubated (37°C , 5% CO_2) in plastic plates for one hour to remove adherent cells. The resulting light-density, nonadherent bone marrow cells were washed once, resuspended in McCoy's 5A medium containing 10% autologous serum (to minimize Fc receptor binding of antibodies in the immunofluorescence and immune rosetting procedures described below), and kept on ice until further use.

Peripheral blood MNC were obtained by centrifugation of diluted blood over Ficoll-Hypaque. After collecting the interface cells and washing twice, the majority of monocytes were depleted by adherence to plastic plates as described above. Nonadherent cells were collected, washed once, resuspended in McCoy's 5A medium containing 10% autologous plasma, and kept on ice.

Preparation of leukemic cells. Leukemic bone marrow and peripheral blood samples were diluted with complete medium and centrifuged over Percoll ($d = 1.050\text{g/mL}$) to remove platelets. Light-density cells, which were then obtained by Ficoll-Hypaque, were washed twice, resuspended in McCoy's 5A medium containing 10% autologous plasma, and kept on ice until further use. Cytospin preparations of the light-density cells were prepared in a Shandon-Elliot cytospin and stained with May-Grunwald-Giemsa, and differential counts were performed on 500 cells. Greater than 85% of the light-density cells were blasts in all acute leukemic samples studied.

Cell separation by FACS IV. Normal bone marrow and peripheral blood nonadherent, light-density cells (15 to 30×10^6), obtained as described, were incubated with previously determined saturating amounts of different monoclonal antibodies for 30 minutes on ice. Control cells (which were used to determine background fluorescence) were incubated with purified mouse IgG (containing all subclasses, Coulter Immunology, Hialeah, FL) except in those experiments where antibody Leu-7 (isotype IgM) was used, in which case control cells were incubated with purified mouse IgM (Coulter). After washing twice the cells were stained with fluorescein isothiocyanate (FITC)-conjugated F(ab')_2 fragments of goat antimouse IgG (Cappel, Malvern, PA) or a purified FITC-labeled antibody against total mouse immunoglobulins (Coulter) when Leu-7 was the incubating antibody. After labeling, the cells were washed twice, resuspended in McCoy's 5A medium containing 5% FCS and 25mmol/L Hepes buffer, and kept on ice, away from light, until FACS IV analysis. Following analysis cells were sorted under sterile conditions into antigen positive (fluorescent cells above control background cells) and negative cell fractions. In some experiments an intermediate fraction consisting of weakly positive staining cells was also collected. To facilitate the separation of antigen positive and negative cells, fluorescence analysis and sorting were performed on a logarithmic scale. Sorted cell fractions were washed twice and resuspended in complete medium for functional analyses (described below) and cytocentrifuge smears.

Cell separation by immune rosetting. Normal bone marrow and peripheral blood light-density, nonadherent cells were incubated with different MoAbs as described above. After washing twice, the cells were resuspended in McCoy's 5A medium containing 10% autologous serum (or plasma) to a cell concentration of 15×10^6 cells/mL and mixed with 1 mL of a 2% solution of bovine red blood

Table 1. Expression of H-25 and H-366 Antigens by Human Cell Lines

Cell Lines	Cell Type	Reference	% Reactivity*	
			H-25	H-366
HSB-2	T Cell lymphoblastic	15	98	98
U937	Monocytic	16	97	96
KG-1	Myeloblastic	17	73	25
HL-60	Promyelocytic	18	80	0
SK-DHL-2	B Cell Lymphoma (CALLA ⁺)	19	31	0
SK-BU-1-Bt(8;14)-1	B Cell Lymphoma (CALLA ⁺)	20	29	0
SK-BU-1-BN-1	EBV-transformed B cell (CALLA ⁻)	20	0	0
SK-CML7-BN-1	EBV-transformed B cell (CALLA ⁻)	21	0	0
SK-CML-7-Bt(9;22)-33†	EBV-transformed B cell (CALLA ⁻)	21	0	0

*Cell lines were reacted with antibodies H-25 and H-366 separately, stained with FITC-conjugated F(ab')₂ fragments of goat antimouse IgG, and analyzed by FACS.

†SK-CML-7-Bt(9;22)-33 contains the 9q;22q translocation (Philadelphia "PH" chromosome). CALLA = Common acute lymphoblastic leukemia antigen. EBV = Epstein-Barr virus.

cells (Hazleton, Denver, PA) coated with affinity purified goat antimouse IgG (Boehringer Mannheim, Indianapolis, IN) by CrCL₃ linkage.²² After centrifugation the tubes were placed on ice for 30 minutes, at which time the pelleted cells were resuspended and rosetted, and nonrosetted cells were separated by centrifugation on a Percoll density solution ($d = 1.080\text{g/mL}$). Nonrosetted cells recovered at the interface and rosetted cells from the pellet (after lysis of indicator bovine RBCs with Tris buffered NH₄Cl) were washed twice and resuspended in complete medium. Efficiency of the immune rosette technique was tested by FACS IV analysis of the nonrosetting cell fractions from peripheral blood. In all cases less than 2% contamination of antigen positive cells were found in the nonrosetting cell fractions regardless of the MoAb used.

Immunofluorescence (FACS IV) analysis of cells from human leukemic samples and cell lines. Light-density cells obtained from the bone marrow and/or peripheral blood of fresh acute leukemic samples as well as established human cell lines were analyzed for H-25 and H-366 antigen expression by indirect immunofluorescence (FACS IV) as described above. The percentage of "positive" cells was calculated by a computer procedure that subtracts the control (background) fluorescence from that of the experimental sample.

Identification of peripheral blood monocytes. Monocytes were identified in the peripheral blood cell fractions obtained by FACS IV sorting and immune rosetting by morphological and histochemical (alpha-naphthyl acetate esterase staining kit, Sigma, St. Louis) analysis of cytocentrifuge smears. The percentage of esterase positive cells was determined by counting 500 cells under oil immersion microscopy.

Progenitor cell assays. Nonadherent, light-density unseparated cells from normal bone marrow and peripheral blood as well as the cell fractions obtained by sorting and immune rosetting were cultured essentially as previously described.⁵ Briefly, for CFU-E and BFU-E, cell cultures consisted of 1 mL of Iscove's modified Dulbecco's medium (IMDM, GIBCO) containing 25% FCS, 0.8% deionized bovine serum albumin, 10% Mo-T cell line-conditioned medium,^{23,24} 10^{-4}M 2-mercaptoethanol, 1 unit of partially purified human urinary erythropoietin (Ep, Toyobo, New York), and methylcellulose at a final concentration of 1.3%. Triplicate cultures were incubated in a humidified atmosphere of 5% CO₂ in air. Day 7 aggregates consisting of greater than eight cells of unequivocally red color were counted as 1 CFU-E. Day 14 red cell aggregates of greater than 50 cells were scored as 1 BFU-E. Peripheral blood erythroid colonies were counted on day 14 only.

The granulocytic/monocytic colony-forming unit (CFU-GM) assay was done exactly as described above for CFU-E and BFU-E,

except that erythropoietin was replaced by an equal volume of IMDM. Aggregates consisting of greater than eight cells were counted on days 7 and 14. Peripheral blood CFU-GM were enumerated on day 14 only. All colony counts were done using an inverted microscope at magnification of 125X.

Calculation of percentage recovery of colonies for each cell fraction obtained by FACS IV and immune rosetting. Total colony recovery for a given type was determined by summing the colonies recovered in each fraction. The number of colonies recovered in each fraction was then expressed as a percentage of the total colonies recovered.

RESULTS

Expression of antigens H-25 and H-366 by normal peripheral blood large granular lymphocytes (LGLs). In three preliminary experiments using the method of indirect immune rosetting, LGLs (where NK cells are known to reside²⁵) were obtained from normal peripheral blood nonadherent MNC by simultaneous immune rosette depletion of T lymphocytes, B lymphocytes, and residual monocytes after reaction with monoclonal antibodies Leu-1, Leu-4, B-1, MY-4, and MY-8. The resulting nonrosetting cell population when examined morphologically consisted predominantly of cells with a slightly indented nucleus and abundant cytoplasm containing azurophilic granules (morphological characteristics ascribed to LGLs²⁵) with <5% alpha-naphthyl acetate esterase positive cells. Figure 1 shows representative FACS IV immunofluorescence profiles of the LGL-enriched cell population obtained after reaction with MoAbs H-25 and H-366 and for comparative purposes with N901, MO-1, and Leu-7, which are also known to react with the majority of LGLs. It can be seen that both H-25 and H-366 antigens are expressed by the LGL-enriched population, although H-25 antigen density is more heterogeneous and considerably higher than H-366 on a substantial number of LGLs. In comparison to N901, H-25 and H-366 antigen densities were considerably higher on many of these cells, while the expression of antigen MO-1 appeared similar to H-366. Leu-7 antigen, like H-25, also displayed broad heterogeneity; however, in contrast to antigens H-25, H-366, MO-1, and N901, which were expressed by approximately 85% of the LGL-enriched population, Leu-7 antigen was expressed by approximately 50%.

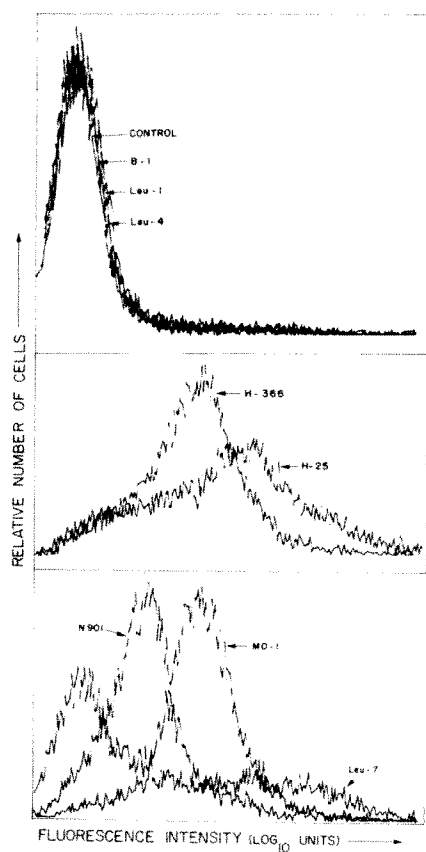


Fig 1. Expression of antigens H-25 and H-366 by LGLs and comparison with antigens N901, MO-1, and Leu-7. LGLs were obtained from peripheral blood by negative selection whereby T lymphocytes, B lymphocytes, and residual monocytes were simultaneously depleted from nonadherent MNC by indirect immune rosetting using MoAbs Leu-1, Leu-4, B-1, MY-4, and MY-8. FACS IV histograms shown here were obtained by indirect immunofluorescence staining after reacting LGLs with each indicated MoAb separately and analyzing 10,000 cells. Background fluorescence staining (control) was obtained by incubating cells with purified mouse IgG (containing all subclasses) or purified mouse IgM (when Leu-7 was the incubating antibody). Note that the number of cells in the LGL enriched cell population reacting with MoAbs B-1, Leu-1, and Leu-4 was $<2\%$, indicating that B and T lymphocytes were efficiently depleted by the immune rosette technique.

FACS IV analysis and sorting of normal bone marrow cells using MoAbs H-25 and H-366. Figure 2 shows representative FACS IV immunofluorescence profiles, before and after sorting, of nonadherent, light-density normal bone marrow cells after reaction with MoAbs H-25 (eight experiments) and H-366 (six experiments). Before sorting, it could be seen that there was a small proportion of bone marrow cells that were positive over background for both antibodies, H-25 being much more pronounced than H-366. Since there was no distinct peak of antigen positive cells for either antibody, a window of cells between 30 to 50 channels (representing an average of 29% and 11% of the H-25 and H-366 antibody-treated cells, respectively) was routinely discarded to minimize overlap between antigen positive and antigen negative cells. In eight experiments,

before sorting, there was a mean of $15\% (\pm 11\%)$ H-25⁺ and $7\% (\pm 2\%)$ H-366⁺ cells. After sorting, FACS IV re-analysis of the H-25⁺ and H-366⁺ cell fractions showed a mean purity of $>85\%$, while H-25 and H-366 cell fractions were always $>98\%$ pure.

Morphology of H-25 positive and H-366 positive bone marrow cells. Differential counts obtained from May-Grunwald-Giemsa-stained cytocentrifuge smears of H-25⁺, H-25⁻, H-366⁺, and H-366⁻ sorted cell fractions are given in Table 2 along with the percent recovery of each of the cell types listed. Blasts, promyelocytes, and monocytes were significantly enriched in the H-25⁺ cell fraction. Based on the total number of cells recovered (sum of the H-25⁺ and H-25⁻ cell fractions), a mean of $76\% (\pm 18\%)$, $88\% (\pm 17\%)$, and $92\% (\pm 16\%)$ of the blasts, promyelocytes, and monocytes respectively were recovered in the H-25⁺ cell fraction. Of the total number of myelocytes, metamyelocytes, and bands (and neutrophils) recovered, $26\% (\pm 31\%)$, $17\% (\pm 33\%)$, and $14\% (\pm 29\%)$ respectively were found in the H-25⁺ cell fraction. These results suggest that antigen H-25 is more strongly expressed by blasts and promyelocytes and sequentially diminishes through myeloid maturation beyond the promyelocyte stage. H-25⁺ cells also included minor proportions of lymphoid (mean = $13\% \pm 6\%$) and nucleated red cells (mean = $18\% \pm 15\%$). Similarly, blasts, promyelocytes, and monocytes were enriched in the H-366⁺ cell fraction; however, the percent recoveries of these three cell types ($54\% \pm 20\%$, $36\% \pm 36\%$, $67\% \pm 27\%$) were lower than that found in the H-25⁺ cell fraction. In contrast to the H-25⁺ cell fraction, essentially all of the myelocytes, metamyelocytes, bands, and neutrophils recovered from sorting with antibody H-366 were found in the H-366⁻ cell fraction. Similar to H-25⁺ cells, H-366⁺ cells also included small numbers of lymphoid (mean = $11\% \pm 5\%$) and nucleated red cells (mean = $26\% \pm 23\%$).

Expression of H-25 and H-366 antigens by hematopoietic progenitor cells from normal bone marrow and peripheral blood. H-25⁺, H-25⁻, H-366⁺, and H-366⁻ sorted bone marrow cell fractions were assayed for erythroid and granulocytic colony-forming units and the results are given in Table 3. For antibody H-25 in the bone marrow of eight individuals tested, a significant but widely variable proportion of day 7 ($39\% \pm 28\%$) and day 14 ($67\% \pm 37\%$) CFU-GM were sorted into the H-25⁺ cell fraction. In contrast, a mean of $99\% (\pm 0.7\%)$ of the CFU-E and $86\% (\pm 17\%)$ of the BFU-E were found in the H-25⁺ cell fraction, suggesting that the expression of antigen H-25 is higher on erythroid progenitor cells (particularly CFU-E) than on granulocytic/monocytic progenitor cells. It should also be noted that while the percent recoveries of CFU-E and BFU-E were similar to the average total cell recovery (43%) in the sorting experiments with antibody H-25, recoveries of day 7 and day 14 CFU-GM were generally about 65% of the expected value.

For antibody H-366 in the bone marrow of six individuals tested, a mean of $97\% (\pm 3\%)$ of the CFU-E and $46\% (\pm 20\%)$ of the BFU-E were sorted into the H-366⁺ cell fraction. In contrast, essentially all of the day 7 and day 14 CFU-GM were found in the H-366⁻ cell fraction, suggesting that the

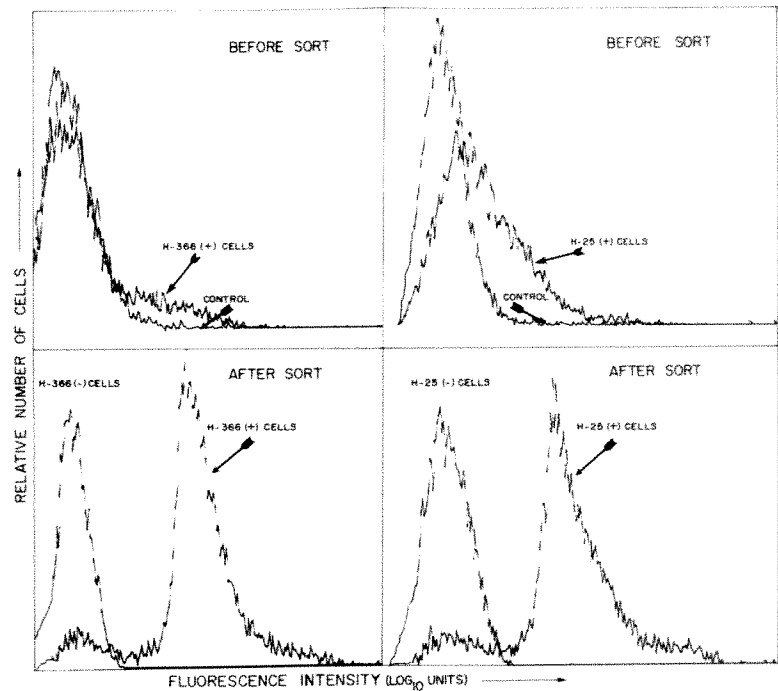


Fig 2. FACS IV analysis of expression of antigens H-25 and H-366 by normal bone marrow cells. Nonadherent, light-density bone marrow cells were separately reacted with MoAbs H-25 and H-366 and separated into H-25⁺ and H-25⁻ and H-366⁺ and H-366⁻ cell fractions using the FACS IV cell sorter (Becton Dickinson). Results are expressed as FACS histograms on a log fluorescence scale with 10,000 cells analyzed. Reanalysis of the sorted cells showed that the purity of the H-25⁻ and H-366⁻ cell fractions were >98% pure, while the H-25⁺ and H-366⁺ cell fractions were >85% pure. Cells from each fraction were then analyzed for morphology and colony assays (see Tables 2 and 3).

expression of antigen H-366, similar to antigen H-25, is relatively high on CFU-E and a significant number of BFU-E but in contrast to H-25 is significantly lower or not expressed by either day 7 or day 14 CFU-GM. The average recoveries of all four types of progenitor cells in the sorting experiments with antibody H-366 were similar to the average total cell recovery (54%).

For comparative purposes, MoAbs Mo-1 and Leu-7 were also reacted with normal bone marrow cells, sorted into Mo-1⁺, Mo-1⁻, Leu-7⁺, and Leu-7⁻ cell fractions and assayed for hematopoietic progenitor cells. The results, also given in Table 3, show that antigens Mo-1 and Leu-7 are not expressed by either erythroid or granulocytic/monocytic

colony-forming units. It should also be noted that, in contrast to the morphological cell profiles seen in the H-25⁺ and H-366⁺ cell fractions (Table 2), neither the Leu-7⁺ nor the Mo-1⁺ cell fractions were enriched for blasts or promyelocytes. Rather, Leu-7⁺ cells consisted of >90% lymphoid-appearing cells, while Mo-1⁺ cells contained >90% mature myeloid cells (myelocytes → neutrophils).

As stated above, a wide variability of day 7 and day 14 CFU-GM were found in the H-25⁺ sorted cell fractions, suggesting that the expression of antigen H-25 on CFU-GM is low. To test this, three experiments were done whereby bone marrow cells were reacted with antibody H-25 and sorted into antigen negative (-), weakly positive (+/-) and

Table 2. Cell Differentials of Bone Marrow Cell Fractions Obtained by (FACS IV) Cell Sorting With H-25 and H-366 Monoclonal Antibodies*

Cell Fraction	Blasts	Pro	Myel	Meta	Band/ Neutrophil	Lymph	Mono	Nucleated Erythrocytes
Unseparated	3 ± 2†	2 ± 1	18 ± 8	14 ± 5	24 ± 13	24 ± 10	3 ± 2	12 ± 5
H-25 Positive	16 ± 6 (76 ± 18)†	8 ± 4 (88 ± 17)	11 ± 10 (26 ± 31)	6 ± 5 (17 ± 33)	8 ± 15 (14 ± 29)	27 ± 12 (13 ± 6)	11 ± 6 (92 ± 16)	10 ± 5 (18 ± 15)
H-25 Negative	1 ± 1 (24 ± 18)	0.2 ± 0.3 (12 ± 17)	8 ± 6 (74 ± 31)	10 ± 6 (83 ± 33)	23 ± 15 (86 ± 29)	41 ± 16 (87 ± 6)	0.4 ± 1 (8 ± 16)	16 ± 8 (82 ± 15)
N = 8								
Unseparated	3 ± 1	2 ± 1	18 ± 7	17 ± 5	25 ± 10	24 ± 10	3 ± 3	7 ± 4
H-366 Positive	23 ± 11 (54 ± 20)	4 ± 4 (36 ± 36)	6 ± 3 (3 ± 2)	4 ± 3 (2 ± 1)	9 ± 6 (5 ± 5)	28 ± 4 (11 ± 5)	14 ± 11 (67 ± 27)	11 ± 6 (26 ± 23)
H-366 Negative	2 ± 1 (46 ± 20)	2 ± 1 (64 ± 36)	15 ± 6 (97 ± 2)	17 ± 6 (98 ± 1)	26 ± 12 (95 ± 5)	32 ± 14 (89 ± 5)	1 ± 1 (33 ± 27)	6 ± 5 (74 ± 23)
N = 6								

*Nonadherent, light-density normal bone marrow cells were separated into H-25 positive, H-366 positive, H-25 negative, and H-366 negative cell fractions by FACS IV cell sorting. Cytocentrifuge smears were stained with May-Grunwald-Giemsa, and 500 cells were counted.

†Values represent the mean % (± 1 SD) of 500 cells counted for N experiments.

‡Values represent the mean % (± 1 SD) of the total respective cell types recovered after sorting.

N = number of experiments.

Pro, promyelocyte; Myel, myelocyte; Meta, metamyelocytes; mono, monocytes.

Table 3. Analysis of Bone Marrow Cell Fractions (Obtained by FACS IV Cell Sorting) for Hematopoietic Colony-Forming Cells*

Cell Fraction	Erythroid colonies 5×10^4 cells		Granulocytic/monocytic (CFU-GM) colonies/ 5×10^4 cells	
	CFU-E	BFU-E	Day 7	Day 14
Unseparated	77 $\pm$ 11†	29 $\pm$ 9	194 $\pm$ 62	39 $\pm$ 10
H-25 Positive	498 $\pm$ 362	190 $\pm$ 248	196 $\pm$ 163	93 $\pm$ 85
	(99 $\pm$ 0.7)‡	(86 $\pm$ 17)	(39 $\pm$ 28)	(67 $\pm$ 37)
H-25 Negative	1 $\pm$ 2	5 $\pm$ 7	97 $\pm$ 80	13 $\pm$ 18
	(1 $\pm$ 0.7)	(13 $\pm$ 17)	(61 $\pm$ 28)	(33 $\pm$ 37)
N = 8				
Unseparated	63 $\pm$ 16	27 $\pm$ 13	189 $\pm$ 60	45 $\pm$ 14
H-366 Positive	984 $\pm$ 584	242 $\pm$ 360	30 $\pm$ 17	15 $\pm$ 12
	(97 $\pm$ 3)	(46 $\pm$ 20)	(1.5 $\pm$ 0.6)	(2 $\pm$ 1)
H-366 Negative	4 $\pm$ 3	19 $\pm$ 17	183 $\pm$ 83	54 $\pm$ 26
	(3 $\pm$ 3)	(54 $\pm$ 20)	(98 $\pm$ 0.6)	(98 $\pm$ 1)
N = 6				
Unseparated	64 $\pm$ 10	26 $\pm$ 3	168 $\pm$ 45	35 $\pm$ 18
Mo-1 Positive	3 $\pm$ 1	1 $\pm$ 1	7 $\pm$ 1	2 $\pm$ 0.3
	(2 $\pm$ 1)	(2 $\pm$ 2)	(3 $\pm$ 1)	(3 $\pm$ 1)
Mo-1 Negative	160 $\pm$ 65	63 $\pm$ 18	329 $\pm$ 47	81 $\pm$ 4
	(97 $\pm$ 1)	(97 $\pm$ 2)	(97 $\pm$ 1)	(97 $\pm$ 1)
N = 2				
Unseparated	81 $\pm$ 2	23 $\pm$ 7	191 $\pm$ 52	32 $\pm$ 3
Leu-7 Positive	3 $\pm$ 3	2 $\pm$ 2	9 $\pm$ 10	5 $\pm$ 6
	(0.3 $\pm$ 0.1)	(0.7 $\pm$ 0.4)	(0.6 $\pm$ 0.6)	(1 $\pm$ 1)
Leu-7 Negative	52 $\pm$ 11	17 $\pm$ 4	131 $\pm$ 35	27 $\pm$ 9
	(99 $\pm$ 1)	(99 $\pm$ 1)	(99 $\pm$ 1)	(99 $\pm$ 1)
N = 2				

*Cell fractions were obtained (after reaction of light-density, nonadherent bone marrow cells with each MoAb separately) by FACS IV cell sorting.

†Value represents the mean number ($\pm$ 1 SD) of colonies per 5×10^4 cells for N experiments.

‡Value represents the mean % ($\pm$ 1 SD) of the total number of colonies recovered after sorting for N experiments.

N = number of experiments.

positive (+) cell fractions. Figure 3 shows representative FACS IV profiles before and after sorting. Cell differentials and the results of progenitor cells assays done on the various cell fractions are given in Table 4 and Fig 4 respectively. Again it is seen that the majority of blasts and promyelocytes were sorted into the H-25⁺ cell fraction along with minor percentages of myelocytes, metamyelocytes, bands, and neutrophils. In contrast, the H-25 (+/-) cell fraction contained minor proportions of blasts and promyelocytes, while substantial numbers of myelocytes, metamyelocytes, bands, and neutrophils were recovered in this fraction. These results again suggest that the expression of antigen H-25 by myeloid cells is inversely related to the state of maturation and therefore is more highly expressed on the more immature blasts and promyelocytes. For the progenitor cell assays an average of 58% ($\pm$ 10%) of day 7 and 53% ($\pm$ 15%) of day 14 CFU-GM were recovered in the H-25 (+/-) cell fraction with lower proportions of both types of granulocytic progenitor cells being found in the H-25⁺ and H-25⁻ cell fractions. In contrast, virtually all the CFU-E were in the H-25⁺ cell fraction, and the great majority of BFU-E also expressed the H-25 antigen (69% $\pm$ 7% were in the H-25⁺ cell fraction and 23% $\pm$ 5% in the H-25 (+/-) cell fraction). These results again suggest that the expression of antigen H-25 by erythroid progenitor cells is high, while the expression by the majority of day 7 and day 14 CFU-GM is relatively low.

Normal peripheral blood CFU-GM and BFU-E, both of which are generally considered to be more primitive than the majority of bone marrow erythroid and granulocytic/mono-

cytic colony-forming units^{26,27} were also examined for the expression of antigens H-25 and H-366. For three separate sorting experiments (results shown in Fig 5), a mean of 86% ($\pm$ 5%) and 39% ($\pm$ 13%) of peripheral blood BFU-E and CFU-GM respectively, were recovered in the H-25⁺ cell fraction while 39% ($\pm$ 22%) of the BFU-E and only 3% ($\pm$ 3%) of the CFU-GM were sorted into the H-366⁺ cell fraction. Thus, the number of peripheral blood CFU-GM and BFU-E that express antigens H-25 and H-366 appears similar to the number found on normal bone marrow BFU-E and 14-day CFU-GM.

Morphological and alpha-naphthyl acetate esterase analysis of the sorted peripheral blood cell fractions showed that both H-25⁺ and H-366⁺ cells consisted predominantly of LGLs and a small number of contaminating monocytes (<9%), while H-25⁻ and H-366⁻ cells were composed of typical small- and medium-sized lymphocytes and <1% monocytes.

The results obtained in the FACS IV experiments were confirmed by studies using the method of immune rosetting to separate normal bone marrow and peripheral blood. Thus, for three separate bone marrow experiments (Fig 6), a mean of 99% ($\pm$ 2%) of the CFU-E and 83% ($\pm$ 20%) of the BFU-E were found in the H-25⁺ immune-rosetted cell fraction, while an average of 39% ($\pm$ 27%) and 77% ($\pm$ 21%) of day-7 and day-14 CFU-GM respectively were recovered in this fraction. For antibody H-366, 96% ($\pm$ 7%), 68% ($\pm$ 41%), 5% ($\pm$ 2%), and 25% ($\pm$ 6%) of the CFU-E, BFU-E, day-7 and day-14 CFU-GM respectively were recovered in the H-366⁺

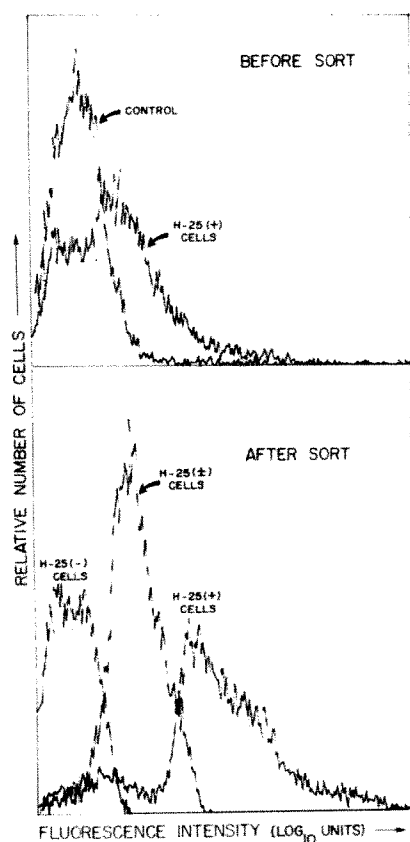


Fig 3. Expression of H-25 antigen by normal bone marrow cells. Nonadherent, light-density bone marrow cells were reacted with H-25 MoAb and separated into the 13% most brightly fluorescent H-25 positive cells (H-25⁺), 27% of the least fluorescent H-25 positive cells (H-25^{+/−}), and H-25 negative (H-25[−]) cell fractions. After sorting, 10,000 cells from each fraction were re-analyzed, and the results here are expressed as FACS histograms. Cell fractions were then analyzed for morphology and colony assays (see Table 4 and Fig 4 respectively).

immune-rosetted cell fraction. For peripheral blood ($n = 3$, Fig 7), 94% ($\pm 3\%$) of the BFU-E and 74% ($\pm 14\%$) of the CFU-GM were found in the H-25⁺ cell fraction while 42% ($\pm 23\%$) and 6% ($\pm 2\%$) of the BFU-E and CFU-GM respectively were H-366⁺. Thus the results obtained by immune rosetting were similar to the FACS IV results with the

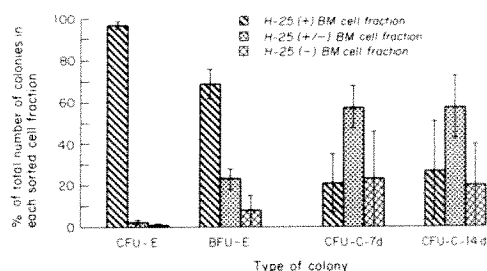


Fig 4. Separation of bone marrow hematopoietic colony-forming cells using MoAb H-25 and FACS IV. Nonadherent, light-density bone marrow cells after reacting with antibody H-25 were separated into H-25 (+), H-25 (+/−), and H-25 (−) cell fractions by FACS IV (see Table 4) and the fractions assayed for hematopoietic colony-forming units. For three separate experiments, H-25 (+) cells were selected as the 13% ($\pm 7\%$) most brightly fluorescent cells, while H-25 (+/−) cells included the 27% ($\pm 10\%$) least fluorescent cells. Bars and vertical lines represent the mean (± 1 SD) of the total number of colonies recovered after sorting. The number of colonies per 5×10^4 unseparated nucleated bone marrow cells were 80 ± 16 CFU-E, 33 ± 20 BFU-E, 179 ± 40 day-7 CFU-GM, and 35 ± 11 day-14 CFU-GM.

exception that a considerably higher number of bone marrow day-14 CFU-GM were found to be H-366⁺ by immune rosetting. Figures 6 and 7 also show that MoAbs Mo-1 and B-1, which served as controls in the immune rosetting experiments, do not react with either normal bone marrow or peripheral blood hematopoietic colony-forming units.

Expression of antigens H-25 and H-366 on acute leukemic cells. In light of the FACS IV and immune rosetting studies with normal bone marrow showing that antigens H-25 and H-366 are expressed by a substantial number of immature myeloid cells, it was of interest to examine the expression of these antigens on fresh leukemic cells. As shown in Table 5, the blast cells from all patients with AML studied were H-25⁺ and H-366⁺, antigen H-25 generally being expressed by a higher number of these blasts. It should be noted that in all these AML patients, the peaks of the fluorescence-intensity profiles obtained with anti-H-366 were noticeably less than those obtained with anti-H-25, indicating that the density of antigen H-25 is higher than antigen H-366 on a substantial number of these blast cells. This observation is similar to what was found for peripheral blood LGLs (Fig 1), where the density of antigen H-25 was

Table 4. Morphological Analysis of Bone Marrow Cell Fractions Obtained by (FACS IV) Cell Sorting With Monoclonal Antibody H-25*

Cell Fraction	Blasts	Pro	Myel	Meta	Band/ Neutrophil	Lymph	Mono	Nucleated Erythrocytes
Unseparated	3 $\pm$ 3†	3 $\pm$ 2	12 $\pm$ 4	12 $\pm$ 3	24 $\pm$ 22	34 $\pm$ 18	2 $\pm$ 2	11 $\pm$ 1
H-25 Positive (+)	18 $\pm$ 7 (67 $\pm$ 16)‡	9 $\pm$ 2 (72 $\pm$ 17)	13 $\pm$ 9 (27 $\pm$ 38)	8 $\pm$ 6 (18 $\pm$ 26)	7 $\pm$ 5 (6 $\pm$ 6)	29 $\pm$ 10 (10 $\pm$ 3)	8 $\pm$ 3 (49 $\pm$ 21)	6 $\pm$ 3 (6 $\pm$ 5)
H-25 Weakly Positive (+/−)	2 $\pm$ 2 (18 $\pm$ 4)	1 $\pm$ 0.2 (25 $\pm$ 12)	22 $\pm$ 11 (48 $\pm$ 25)	20 $\pm$ 7 (47 $\pm$ 26)	18 $\pm$ 10 (43 $\pm$ 39)	20 $\pm$ 14 (13 $\pm$ 3)	2 $\pm$ 1 (40 $\pm$ 17)	14 $\pm$ 9 (29 $\pm$ 17)
H-25 Negative (−)	0.7 $\pm$ 0.7 (15 $\pm$ 18)	0.007 $\pm$ 0.1 (3 $\pm$ 6)	5 $\pm$ 6 (25 $\pm$ 30)	7 $\pm$ 8 (35 $\pm$ 40)	23 $\pm$ 22 (52 $\pm$ 46)	50 $\pm$ 35 (77 $\pm$ 5)	0.4 $\pm$ 0.4 (11 $\pm$ 13)	13 $\pm$ 3 (65 $\pm$ 22)

*Nonadherent, light-density normal bone marrow cells were separated by FACS IV into H-25 positive (+), H-25 weakly positive (+/−), and H-25 negative (−) cells. Cell differentials were obtained from May-Grunwald-Giemsa stained cytocentrifuge smears.

†Values represent the mean (± 1 SD) of 500 cells counted for three separate experiments.

‡Values represent the mean (± 1 SD) of the total respective cell types recovered for three experiments. See legend to Table 2 for description of cell types listed.

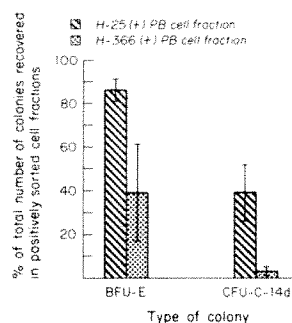


Fig 5. FACS IV separation of peripheral blood hematopoietic colony-forming cells using MoAbs H-25 and H-366. FACS IV-sorted cell fractions were obtained after reaction of nonadherent, light-density peripheral blood MNC with MoAbs H-25 and H-366 separately. Bars and vertical lines represent the mean % (± 1 SD) of the total number of colonies recovered after sorting for three separate experiments. The number of colonies per 3×10^5 unseparated MNC were 39 ± 18 BFU-E and 11 ± 8 CFU-GM.

also found to be higher than H-366 on peripheral blood LGLs and also for normal bone marrow (Table 2) where a higher number of blasts were found to be H-25⁺.

Both antigens were also expressed by the blast cells from two patients with T-ALL; however, in both cases antigens H-25 and H-366 were equally expressed. In one pre-B, Calla⁺ ALL patient, 60% and 34% of the blasts were positive for H-25 and H-366 respectively. Finally, in another pre-B, Calla⁺ ALL patient, 25% and 14% of the cells were H-25⁺ and H-366⁺ respectively. It must be emphasized, however, that in both of these pre-B, ALL cases, both H-25 and H-366 positive cells were weakly fluorescent.

Expression of H-25 and H-366 antigens on human cell lines. Table 1 gives the results obtained by testing some human cell lines for reactivity with antibodies H-25 and H-366. The parent T-ALL cell line, HSB-2, and the monocytic cell line, U937 were both strongly reactive (>95%) with H-25 and H-366. Furthermore, both antigens were found to

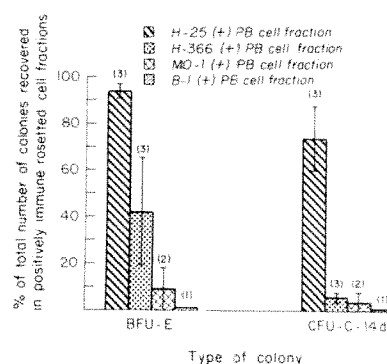


Fig 7. Separation of peripheral blood hematopoietic colony-forming cells using MoAbs and immune rosetting. Nonadherent, light-density peripheral blood MNC were reacted with MoAbs H-25, H-366, Mo-1, and B-1 separately and separated into antigen positive and negative cell fractions by indirect immune rosetting. Bars and vertical lines represent the mean % (± 1 SD) of the total number of colonies recovered after rosetting. The number of experiments are given in parentheses. The average percent recoveries of CFU-GM and BFU-E were similar to the average total cell recovery (78%). The number of colonies per 3×10^5 unseparated MNC were 53 ± 17 BFU-E and 12 ± 2 CFU-GM.

be equally expressed by these two cell lines. Both the myeloblastic cell line, KG-1, and the promyelocytic cell line, HL-60, were H-25⁺; however, the density of antigen H-25 was lower on KG-1 and HL-60 cells than on HSB-2 and U937 cells. In addition, while KG-1 cells contained 25% of weakly staining H-366⁺ cells, the HL-60 cell line did not express this antigen. Of the B cell lines tested, all Calla⁻, Epstein-Barr virus (EBV)-transformed B cell lines (including one that contained the Philadelphia [Ph¹] chromosome^{21,28}) were found to be negative for H-25 and H-366, while the two human B cell lymphoma cell lines, SK-DHL-2 and SK-BU-1-Bt(8;14)-1, both of which are Calla⁺ and

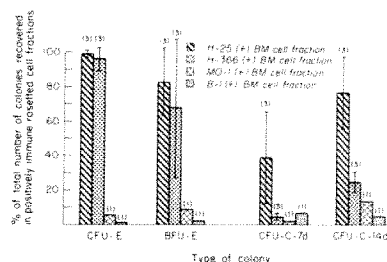


Fig 6. Separation of bone marrow hematopoietic colony-forming cells using MoAbs and immune rosetting. Nonadherent, light-density bone marrow cells, after separately reacting with MoAbs H-25, H-366, Mo-1, and B-1, were separated into antigen positive and negative cell fractions by indirect immune rosetting using bovine RBC coated with goat antimouse IgG. Bars and vertical lines represent the mean % (± 1 SD) of the total number of colonies recovered after rosetting. The number of experiments are given in parentheses. For these experiments the average percent recoveries of colony-forming cells were similar to the average total cell recovery (74%). The number of colonies per 5×10^4 unseparated bone marrow cells were 88 ± 25 CFU-E, 44 ± 14 BFU-E, 210 ± 116 day-7 CFU-GM and 37 ± 18 day-14 CFU-GM.

Table 5. FACS IV Analysis of Cells From Acute Leukemic Patients With H-25 and H-366 Monoclonal Antibodies

Patient	Diagnosis†	(FAB Classification)	% Reactivity*	
			H-25	H-366
SS	AML	(M2)	66	42
EM	AML	(M2)	67	28
HR	AML	(M2)	94	89
CM	APL	(M3)	84	81
CH	APL	(M3)	84	64
GA	AMOL	(M5A)	46	23
JD	AMOL	(M5B)	54	50
CC	T-ALL		88	89
MB	T-ALL		65	56
JV	Pre-B, ALL		25	14
DM	Pre-B, ALL		60	34

*Light-density bone marrow or peripheral blood cells from leukemic patients were reacted with antibodies H-25 and H-366 separately, washed, stained with FITC-conjugated F(ab')₂ fragments of goat antimouse IgG, and analyzed by FACS IV. >85% of the light-density cells were blasts (determined by May-Grunwald-Giemsa stain morphology) in all cases.

†AML, acute myelogenous leukemia; APL, acute promyelocytic leukemia; AMOL, acute monocytic leukemia; T-ALL, T cell acute lymphocytic leukemia.

contain the t(8;14) translocation,^{19,20,29} each contained approximately 30% of weakly fluorescent H-25⁺ cells.

DISCUSSION

In the present studies we have confirmed the previous observations of Bai et al⁷ showing that antigens H-25 and H-366 are expressed by human NK cells and have extended these observations to also show that both these antigens are additionally expressed by normal human bone marrow immature cells of the myeloid series. These cells included the majority of blasts and promyelocytes, with sequentially fewer of the more mature cells of the myeloid series. Both antigens were also found on the majority of bone marrow monocytes, which is consistent with previous studies⁷ demonstrating that they are expressed by a substantial number of peripheral blood monocytes.

Of particular interest in the results reported herein was our observation of the distribution of antigens H-25 and H-366 on erythroid and granulocytic/monocytic colony-forming units. H-25 was found to be expressed by all bone marrow CFU-E and the majority of the more primitive BFU-E from both bone marrow and peripheral blood. CFU-GM expression of antigen H-25, on the other hand, was more variable, with significant numbers of both day-7 and day-14 CFU-GM being found both in the positive and negative fractions. The results of the cell-sorting experiments in Fig 4 suggest that this variability may be due to low antigen expression, since substantial numbers of both day-7 and day-14 CFU-GM were sorted into an H-25 weakly positive cell fraction. A significant number of CFU-GM from peripheral blood was also found to be H-25⁺; however, the cell-sorting method detected fewer than that found by immune rosetting. This disparity again may be explained by the fact that in the sorting experiments a window of cells, which were routinely gated out to minimize overlap between antigen positive and antigen negative cells, may include a proportion of H-25 weakly positive CFU-GM that can be detected by the method of immune rosetting.

Similar to H-25, we also found antigen H-366 to be expressed by virtually all bone marrow CFU-E, but, in contrast to H-25, antigen H-366 was found on a lower number (approximately 50%) of BFU-E from both bone marrow and peripheral blood. Also in contrast to antigen H-25, H-366 was not expressed by any significant number of CFU-GM from either bone marrow or peripheral blood.

In testing a variety of human cell lines as well as fresh blast cells from a limited number of leukemic patients in the present studies, our results (Tables 1 and 5) indicate that antigens H-25 and H-366 are expressed both in T-ALL and myeloid leukemias; however, it should be noted that the promyelocytic cell line, HL-60, while being H-25⁺, did not express the H-366 antigen. The absence of the latter antigen on HL-60 cells is an interesting observation, since we did find H-366 to be expressed by a significant number (mean = 36%, Table 2) of promyelocytes from normal bone marrow. It may be that the more differentiated H-366 antigen negative promyelocytes (since H-366 antigen is found on some promyelocytes but not on myelocytes, Table 2) represent the normal counterpart to HL-60 promyelocytic cells.

With regard to their weak expression by only those B cell lines that are Calla⁺ (Table 1) as well as being weakly expressed on a proportion of fresh blast cells from two Calla⁺, Pre-B ALL patients (Table 5), these results, taken together, suggest that antigen H-25 (and possibly H-366) may also be weakly expressed on immature B cells having a Calla⁺ phenotype; however, a more extensive number of B cell lines as well as patients with hematologic malignancies of B cell origin will have to be tested to confirm this.

Recently several other MoAbs that react with many or all NK cells have been described. To date these include Leu-7,¹⁴ N901,¹³ Mo-1,⁸ B73.1,³⁰ Leu-11,³¹ Lyt-3, 3A-1, and OKT10.³² However, the previously reported characterization of antigens H-25 and H-366 with regard to their respective molecular weight and cellular distribution indicates that MoAbs H-25 and H-366 are distinct from these other reported antibodies.⁷ Moreover, the results reported in the present studies further indicate that antigens H-25 and H-366 are of particular interest since they were found to be additionally expressed on a significant number of bone marrow immature myeloid cells, including hematopoietic colony-forming units (particularly CFU-E and BFU-E). Of these other NK cell-reacting MoAbs mentioned above, only OKT10 has been found to react with hematopoietic precursor cells. Thus, Sieff et al,³³ in cell-sorting experiments, have shown the OKT10 antigen to be expressed by the majority of bone marrow BFU-E, a proportion of CFU-E, and CFU-GM, as well as myeloblasts, promyelocytes, and some myelocytes. It was also shown to be expressed by >95% thymocytes and bone marrow TdT⁺ cells.

Thus, while OKT10 (similar to H-25 and H-366) is expressed by LGLs, immature myeloid cells, and thymocytes, it can be distinguished from H-25 and H-366, since the OKT10 antigen has been reported to have a molecular weight of 45 kilodaltons,³⁴ whereas H-25 and H-366 antigens have molecular weights of 96 kilodaltons and 53 kilodaltons respectively.⁷

Since fresh peripheral blood NK cells as well as in vitro-established cloned NK cell lines³⁵ express several cell surface antigens that are also found on thymocytes, T cells (T11, T8), and cells of myeloid lineage (Mo-1, B73.1), their origin and developmental lineage is still controversial. Our observations that antigens H-25 and H-366 are expressed by all three types of cells confirm the uncertainty of NK cell lineage. However, the present finding that antigens H-25 and H-366 are also expressed by bone marrow myeloid precursor cells provides additional evidence^{36,37} that circulating NK cells, like other lymphoid and myeloid cells, are bone marrow derived.

Although the functional roles that antigens H-25 and H-366 have on NK cells are presently unknown, previous studies⁷ have shown that neither antibody blocks NK or K cell activity. With regard to their presence on bone marrow immature myeloid cells, their patterns of expression make it conceivable that they may be involved in proliferation and differentiation events. It is well known, for example, that both monocytes and NK cells exert a regulatory role on the proliferation and differentiation of hematopoietic progenitor cells^(1-3,38-40) and, since both monocytes and NK cells express both antigens, it is possible that antigens H-25 and H-366

may mediate a complex network of cell-cell interactions between monocytes, NK cells, and hematopoietic precursor cells. Experiments to test this hypothesis are currently in progress in our laboratory.

Finally, with appropriate purification strategies, MoAbs H-25 and H-366 may prove to be very useful reagents for isolating immature myeloid bone marrow cells to study hematopoietic cell proliferation and differentiation once

mature NK cells and monocytes have been depleted using other readily available anti-NK and antimonocyte reagents.

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A Monoclonal Antibody Detecting a Novel Antigen Expressed in the HTLV-I-Infected Cells

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A monoclonal antibody, FTF 148, was prepared by hybridizing murine myeloma cells (NS-1) and spleen cells of BALB/c mice immunized with cultured cells derived from an adult T cell leukemia (ATL) patient (KUT-2 cells). This monoclonal antibody reacted with all of the human T cell leukemia virus I (HTLV-I)-infected cell lines tested but did not react with other T cell lines derived from acute lymphocytic leukemia, Epstein-Barr virus-transformed B cell lines, or an erythroleukemic cell line. This monoclonal antibody was not directed to viral antigens because it reacted equally well with almost all KUT-2 and MT-1 cells, only 1% to 3% of which were ATL-associated antigen-

positive. In contrast to interleukin 2 receptors expressed on both ATL cells and normal phytohemagglutinin-stimulated blasts, this antigen was not expressed on the latter cells. The antigen, mainly expressed on the cell membrane, was analyzed by metabolic labeling with ^3H -leucine and surface labeling with ^{125}I followed by cell lysis and immunoprecipitation with the FTF 148 antibody. The findings obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that p50 and p74 proteins were specifically precipitated and the antigen was also different from the product of the Xs gene of HTLV-I.

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ADULT T CELL LEUKEMIA (ATL) is a particular type of T cell malignancy first reported by Takatsuki et al.^{1,2} Clinical features of ATL include high white cell counts, frequent skin lesions, hepatosplenomegaly, lymphadenopathy, and a rapidly fatal terminal course. The human type C retrovirus human T cell leukemia virus I (HTLV-I) was first isolated from cultured T cells derived from a patient with a cutaneous T cell lymphoma (mycosis fungoides) and subsequently isolated from cell lines derived from ATL patients.³⁻⁵ Seroepidemiological and molecular biologic analyses suggested that HTLV-I is associated with ATL and plays a causative role in the leukemogenesis of ATL.⁵ However, studies revealed that this virus does not have cell-derived oncogenes and the integration site varies from one patient to another.^{6,7} Therefore, the mechanism of leukemogenesis by HTLV-I still remains to be elucidated. The HTLV-I genome has been shown to carry the Xs gene, and its products (P_x) enhance the transcription of its own long terminal repeat.⁸⁻¹⁰ This protein might also activate cellular gene expression, and this activation might be associated with T cell transformation.

To identify the cellular gene product necessary to maintain the proliferation of ATL cells, we tried to obtain monoclonal antibodies that react specifically with ATL-

derived cells irrespective of the production of HTLV-I virus. We obtained a monoclonal antibody, FTF 148, for this purpose. The antigen detected with this monoclonal antibody proved to be different from the interleukin 2 (IL 2) receptor, the Ia-like antigen, viral antigens and the Xs gene product. We report the specificity of the monoclonal antibody together with some characterization of the new antigen.

MATERIALS AND METHODS

Cells. The HTLV-I-infected cell lines KUT-1 and KUT-2 were established from the peripheral blood lymphocytes (PBL) of ATL patients by cultivation in RPMI 1640 medium supplemented with 10% human cord serum.¹¹ UW-4 is the IL 2-dependent cell line derived from PBL of an ATL patient. Three T cell lines (MT-1, MT-2, and HUT-102) and three B cell lines derived from ATL patients (KUT-3, ATL-B-1, and ATL-B-2) were also used as HTLV-I-infected cell lines. Five cell lines (MT-1, MT-2, KUT-1, KUT-2, and HUT-102) were cloned lines. The integration of the proviral DNA was confirmed in many cell lines (MT-1, MT-2, KUT-1, KUT-2, KUT-3, and HUT-102). As negative-control target cells we used three T cell lines derived from acute lymphocytic leukemia (CCRF-CEM, HSB-2, and MOLT-4), two Epstein-Barr virus (EBV)-transformed B cell lines, another B cell line (Raji), and the K-562 cell line. In some experiments, PBL from healthy adults were separated with Ficoll-Paque and the cells were cultured with phytohemagglutinin-P (PHA-P) or with concanavalin A (Con A) (2 $\mu\text{g}/\text{mL}$) for 48 hours and were used as negative-control target cells. These cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. This medium is referred to as RPMI complete medium.

Preparation of monoclonal antibodies. BALB/c mice were immunized twice at 1-week intervals by intraperitoneal (IP) injections of 1.0×10^7 KUT-2 cells followed by a booster immunization with the same dose of KUT-2 cells using the same route. Four days after the last immunization, the mice were sacrificed, and their spleen cells (1×10^8 cells) were fused with mouse myeloma cells (NS-1, 2×10^7 cells) using 45% polyethylene glycol 4000 (Merck, Darmstadt, West Germany). The cells were seeded in 96-well flat-bottomed tissue culture plates (No. 25860, Corning Glass Works, Corning, NY) at a concentration of 1.0×10^5 spleen cells per well with normal spleen cells from BALB/c mice (3×10^5 cells/well) as feeder cells. The cells were cultured in RPMI complete medium supplemented with hypoxanthine (100 $\mu\text{mol}/\text{L}$), thymidine (16 $\mu\text{mol}/\text{L}$) and aminopterin (0.1 $\mu\text{mol}/\text{L}$), and approximately 1,200 hybridoma clones were established. The clones that reacted specifically with the ATL-derived cell line were selected by the

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following screening program. In the first screening step, the culture supernatants (CS) of hybridoma clones that reacted with the majority of acetone-fixed KUT-2 cells were selected by an indirect immunofluorescence method. In the next step, the CS reactive with either EBV-transformed B cells or PHA-stimulated PBL were eliminated. In the third step, the CS reactive with PBL from an ATL patient cultured for four hours were selected. In the last screening step, the CS reactive with non-ATL T cell lines (HSB-2, CEM, and MOLT-4) were eliminated. Two hybridoma clones were selected by this screening program (FTF 148 and FTG 193). Although these clones were prepared independently, the staining patterns of the ATL-derived cell lines under indirect immunofluorescence were almost identical. The findings obtained using FTF 148 are reported here.

Immunoelectron microscopic studies. The cultured cells were harvested and washed three times with Dulbecco's phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) (100 $\mu\text{g}/\text{mL}$). The cells were pelleted by centrifugation and fixed in periodate-lysine-paraformaldehyde (PLP) according to the method of Mclean and Nakane.¹² After a two-hour fixation at 4 °C, the pellets were washed with PBS containing 10% sucrose and further washed with an increasing series of sucrose in PBS. Finally they were placed in PBS containing 20% sucrose and 10% glycerol. The fixed cells were embedded in an OTC compound (Miles Laboratories, Naperville, IN) and frozen in liquid nitrogen. Ten-micrometer-thick frozen sections were placed on slides, overlaid with diluted monoclonal antibody (2,000-fold diluted ascites), and incubated in a humidified chamber at room temperature for two hours. Then the sections were reacted with biotinylated horse antimouse IgG for two hours followed by incubation with both an avidin-biotinylated peroxidase complex and a peroxidase-mouse antiperoxidase complex for two hours at room temperature. The sections were then fixed in 2% glutaraldehyde for ten minutes at room temperature, washed with PBS, and incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. After postfixation in OsO_4 for one to two hours, they were dehydrated in a graded series of alcohol every five to ten minutes and embedded in inverted gelatin capsules filled with epoxy resin. The sections were cut on an LKB Ultratome (Stockholm) with a diamond knife. The ultrathin sections were stained with uranyl acetate and lead citrate. Both stained and unstained sections were examined with a JEM 100 CX electron microscope (Jeol, Inc, Peabody, MA).

Radioactive labeling and immunoprecipitation. KUT-2 cells were labeled by two independent methods: cell surface antigen was labeled with ^{125}I by the lactoperoxidase method,¹³ and proteins were metabolically labeled with ^3H -leucine for 16 hours (10 $\mu\text{Ci}/\text{mL}$ in leucine-free RPMI 1640 medium supplemented with 10% FCS). The labeled cells (1×10^7 cells) were washed three times with PBS and then treated for ten minutes at 4 °C with 2 mL of lysis buffer (50 mmol/L Tris-HCl, pH 8.0; 100 mmol/L NaCl; 0.5% sodium deoxycholate). The lysates were sonicated and were cleared by centrifugation at 7,000 g for 30 minutes at 4 °C. The samples (200 μL) were incubated with 6 μg IgG of FTF 148 or with normal mouse IgG (50 μL) for four hours at 4 °C and for another two hours with 120 μg IgG of rabbit antimouse Ig (120 μL). They were further incubated with 200 μL of a 40% suspension of protein A-Sepharose 4B for 30 minutes at 4 °C, and the gel was washed twice with the lysis buffer, twice with PBS containing 0.1% BSA and further washed twice with BSA-free PBS. The immunoprecipitates were suspended in 200 μL of the electrophoresis sample buffer (0.0625 mol/L Tris-HCl, pH 6.8; 2.3% sodium dodecyl sulfate [SDS]; 5% 2-mercaptoethanol) and heated at 50 °C for two hours. After centrifugation, the supernatants (30 μL) were introduced into the slots of analytic SDS-polyacrylamide gel and separated at a constant voltage (100 V). After electrophoresis, the gels were stained

and processed for autoradiography (^{125}I surface label) and fluorography (^3H -leucine metabolic label).

Immunofluorescence assay. For the assay of the reactivity of the FTF 148 antibody, an indirect immunofluorescence method was used. The smeared target cells were fixed with cold acetone (-20 °C) for five minutes and incubated in a moisture chamber for 30 minutes at room temperature with CS or Ig partially purified from the ascites of mice that had been injected with the hybridoma cells. The slides were washed three times with PBS containing BSA (100 $\mu\text{g}/\text{mL}$) and incubated for 30 minutes with fluorescein isothiocyanate (FITC)-conjugated Ig of rabbit antimouse Ig absorbed with human Ig conjugated to Sepharose 4B and human spleen acetone powder. After two washings with PBS containing BSA, the slides were again washed twice with BSA-free PBS and mounted in 95% glycerin. In some experiments viable cells were stained by the same procedure, washed as before, suspended in Dulbecco's PBS and observed under a fluorescence microscope.

Partial purification of IgG from ascites. The cloned hybridoma cells were implanted IP in pristane-primed BALB/c mice, and the ascites fluids were collected. The IgG fraction was prepared from ascites by repeated precipitation with 45% saturated ammonium sulfate (pH 6.2). The resulting precipitates were dialyzed against PBS.

Immunoglobulin subclass determination. The CS of the hybridoma cells were concentrated tenfold with Minicon B15 (Amicon Corp, Danvers, MA), and the immunoglobulin subclass was determined by the double-immunodiffusion method using isotype-specific rabbit antimouse immunoglobulin.

Tunicamycin treatment of KUT-2 cells. KUT-2 cells (1×10^6) were incubated for 16 hours in RPMI complete medium in the presence of tunicamycin (0.8 $\mu\text{g}/\text{mL}$) and were used for the viable cell immunofluorescence study. For metabolic labeling of the tunicamycin-treated cells, the cells were incubated on leucine-free RPMI 1640 medium supplemented with 10% FCS and tunicamycin (0.8 $\mu\text{g}/\text{mL}$) for one hour, then ^3H -leucine (10 $\mu\text{Ci}/\text{mL}$) was added to the medium, and the incubation was continued for 15 hours.

Chemicals and reagents. The monoclonal antibodies against the human IL 2 receptor (HIE1, H-31, and H-A26)¹⁴ were kindly provided by Dr Y. Hinuma and Dr K. Sugamura. Tunicamycin, an inhibitor of N-linked glycosylation,¹⁵ was kindly provided by Dr G. Tamura. The FITC-conjugated IgG of rabbit against mouse IgG was prepared in our laboratory. The other antibodies were isotype-specific rabbit antimouse immunoglobulin obtained from Zymed Laboratories (San Francisco) and peroxidase mouse antiperoxidase (mouse PAP) from E-Y Laboratories, San Mateo, CA. Avidin-biotinylated peroxidase was purchased from E-Y Laboratories and protein A-Sepharose 4B from Pharmacia P-L Biochemicals, Uppsala, Sweden. ^3H -leucine and ^{125}I were purchased from New England Nuclear, Boston. RPMI 1640 was obtained from GIBCO, Grand Island, NY; FCS from M.A. Bioproducts, Walkersville, MD; Con A and PHA-P from Pharmacia P-L Biochemicals; DAB from Dojin Chemical Institute, Japan, and Ficoll-Paque from Pharmacia P-L Biochemicals. Sodium deoxycholate was purchased from Nakarai Chemicals, Japan.

RESULTS

Preparation and reactivity of the FTF 148 monoclonal antibody. From approximately 1,200 hybridoma clones, two clones (FTF 148 and FTG 193) were selected by the screening program presented in Materials and Methods. Although the immunoglobulin isotypes of FTF 148 and FTG 193 were IgG₁ and IgA respectively, the staining pattern of the immunofluorescence was almost the same, and the antigen reactive with FTF 148 antibody was characterized.

First, the reactivity of this antibody with various kinds of human lymphoid cell lines was examined by the indirect immunofluorescence method using viable cell or acetone-fixed smeared cells (Table 1). The FTF 148 antibody reacted with all of the HTLV-I-infected cell lines tested. This antibody, however, did not react with other T cell lines derived from acute lymphocytic leukemia (HSB-2, CEM, and MOLT-4), EBV-transformed B cell lines, or an erythroleukemic cell line (K562). The only exception was the Raji cell line in which approximately 1% of the cell population was positively stained by a membrane immunofluorescence method. The possibility that this antigen, mainly expressed on cell membranes, is identical with the IL 2 receptor molecule expressed in abundance in HTLV-I-infected cells was ruled out because normal PBL stimulated with PHA or Con A reacted with a monoclonal antibody against the IL 2 receptor (HIEI, H-31, and H-A26) but did not react with the FTF 148 monoclonal antibody (Table 2). The expression of the antigen detected with the FTF 148 monoclonal antibody correlated well with the integration of HTLV-I proviral DNA in the cells but did not correlate with the expression of the ATL-associated antigen (ATLA) detected with anti-ATLA-positive human serum (Table 3). As presented in Table 3, the FTF 148 antibody reacted with more than 90% of the KUT-2 and MT-1 cells, only 1% to 3% of the cells of which expressed the ATLA. This antibody also reacted with more than 90% of the KUT-1 cells in which ATLA-positive

Table 1. Reactivity of the FTF 148 Antibody With Human Hematopoietic Cell Lines

Cell Line	Percentage of Fluorescence-Stained Cells With FTF 148
ATL-derived T cell lines	
MT-1	>90
MT-2	>90
HUT-102	>90
KUT-1	>90
KUT-2	>90
UW-4	>90
ATL-derived B cell lines	
KUT-3	80
ATL-B-1	75*
ATL-B-2	80*
Non-ATL T cell lines	
HSB-2	0
CCRF-CEM	0
MOLT-4	0
Non-ATL B cell lines	
Raji	1-2
EBV-1	0
B-ALL-1	0*
B-CLL-1	0*
Non-ATL non-T/non-B cell line	
K-562	0

The percentage of positive cells was determined by an indirect immunofluorescence method using acetone-fixed smeared cells. FITC-conjugated IgG of rabbit against human IgG or mouse IgG was used as second antibodies.

*Determined by the surface immunofluorescence method.

Table 2. Reactivity of Lectin-Stimulated PBL With FTF 148 Antibody and Monoclonal Antibodies Against the IL 2 Receptor

Antibody	Percentage of Fluorescence-Stained Cells in		
	Nonstimulated PBL*	PHA-Stimulated PBL†	Con A-Stimulated PBL‡
FTF 148	0	0	0
HIEI	<2	40-60	25-40
H-31	<2	45-60	20-35
H-A26	<1	40-60	20-40

The percentage of positive cells was determined by an indirect immunofluorescence method using viable cells. Three to five healthy adult PBL were used for each antibody.

*PBL cultured for 48 hours without lectin stimulation. Fresh PBL were also stained with FTF 148 antibodies (four cases).

†PBL stimulated with PHA-P (2 µg/mL) for 48 hours.

‡PBL stimulated with Con A (2 µg/mL) for 48 hours.

cells were not detected. Furthermore, this antibody reacted with almost all of the PBL that had been taken from an ATL patient and cryostocked after a four-hour culture although ATLA-positive cells were still very rare. Because anti-ATLA-positive antiserum mainly detects *env* and *gag* protein, the FTF 148 antibody may not be directed to the viral antigen of HTLV-I.

The reactivity of this antibody to fresh PBL obtained from ATL patients was examined by a viable cell surface immunofluorescence method. In some cases a significant percentage of PBL reacted with this antibody (Table 4). In most cases, the ATL cells in the peripheral blood did not express this antigen *in vivo* thereby making it necessary to elucidate the mechanism of expression of this antigen in relation to the expression of the HTLV-I genome.

Localization of the antigen in ATL-related cultured cells. The acetone-fixed KUT-2 cells demonstrated a strongly positive immunofluorescence reaction mainly on the cell membrane and a relatively weak reaction in the cytoplasm. The antigenic determinant of this antigen is exposed to the external lipid bilayer of the plasma membrane because viable cells reacted with this antibody very strongly. Immunoelectron microscopic analysis of PLP-fixed cells using an immunoperoxidase technique revealed that in MT-2 cells this monoclonal antibody reacted with some components of the plasma membrane (Fig 1). The linear pattern of a positive peroxidase reaction was usually observed, and the

Table 3. Reactivities of the FTF 148 Antibody and Anti-ATLA-Positive Human Serum With ATL-Derived T Cell Lines

Cell Line	Percent of Immunofluorescence-Stained Cells With	
	Anti-ATLA	FTF 148
MT-1	1-3	>90
MT-2	90	>90
HUT-102	ND	>90
KUT-1	0	>90
KUT-2	1-3	>90
UW-4	1-2	>90

The percentage of positive cells was determined by an indirect immunofluorescence method using acetone-fixed smeared cells. FITC-conjugated IgG of rabbit against human or mouse IgG was used as a second antibody.

Abbreviation: ND, not determined.

Table 4. Reactivity of the FTF 148 Antibody With Fresh PBL of ATL Patients

Source of PBL	No. of Cases	Percentage of Positive Cells in PBL			
		0-1	1-5	5-10	>10
ATL patients	18	7	3	4	4*
Healthy adults of nonendemic area	11	11	0	0	0
Blood donors in endemic area	27	24	1	2†	0

PBL were separated from peripheral blood with Ficoll-Paque, and the percentage of positive cells was determined by an indirect immunofluorescence method.

*More than 40% of PBL were positively stained in two out of four cases.

†Anti-ATLA-positive adults. Another seropositive adult in this group did not have any positively stained cells in PBL.

localization of the reaction was not correlated to that of virus particles that were abundant in the vesicular structure in the cytoplasm and also in the extracellular space. In HUT-102 cells, not only was the plasma membrane positively stained by an immunoperoxidase method, but also the rough endoplasmic reticulum and some parts of the nuclear membrane were positively stained (Fig 2). These results coincide well with the findings obtained using the acetone-fixed smeared cells.

Characterization of the antigen detected with the FTF 148 antibody. For biochemical analysis, KUT-2 cells were labeled with ^3H -leucine for 16 hours, processed as described in Materials and Methods and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% slab gels. The fluorographic patterns of the material immunoprecipitated with the FTF 148 antibody and the material immunoprecipitated with normal mouse IgG revealed that two proteins were specifically precipitated (Fig 3). The monoclonal antibodies against other cellular components (eg, antihuman *c-myc*, anti-Px) did not precipitate this antigen. These proteins had apparent molecular weights (mol wt) of 74,000 and 50,000. Treatment of KUT-2 cells with tunicamycin (0.8 $\mu\text{g}/\text{mL}$) so

as to decrease the ^3H -glucosamine incorporation into the cellular proteins by 95% did not reduce the apparent mol wt of these proteins, which suggests that these proteins did not have a long N-linked carbohydrate chain. The tunicamycin-treated cells had as many antigenic determinants as non-treated cells on the cell membrane as evidenced by a surface immunofluorescence study.

From the ^{125}I surface-labeled material, these two proteins were also specifically precipitated, thus suggesting that at least a part of these proteins was exposed to the exterior of the plasma membrane (Fig 4). The relationship between these two proteins is still unclear. These two proteins may form a complex on the plasma membrane, and the antibody may be directed to one of them.

This antigen is not the product of the Xs gene of HTLV-I (Px) because the latter protein has been reported to be distributed in the nucleus and cytoplasm.^{16,17} We have recently obtained a monoclonal antibody against the product of a fourth open reading frame of the Xs gene (Px IV) and confirmed this result (unpublished observations). The analysis of the detailed structure and the function of this antigen, supposedly a product of a cellular gene activated by a transacting viral gene product, is now in progress.

DISCUSSION

ATL is considered to be caused by an HTLV-I infection based on proviral DNA analysis and seroepidemiological studies, but the relationship between viral infection and leukemogenesis is still unclear. Recently Sodroski et al showed that there exists a transacting factor in HTLV-I-infected cells and this factor may be the product of the Xs region of the HTLV-I genome.¹⁰ This factor is now believed to promote cellular gene activation, and the product of the latter is responsible for cell transformation. In this respect, it is important to analyze the cellular gene products newly expressed by the infection of HTLV-I. The HTLV-I-infected leukemic cells generally have a surface phenotype characteristic of mature helper T cell and express the IL 2 receptor in abundance and HLA-DR antigens.¹⁸⁻²⁰

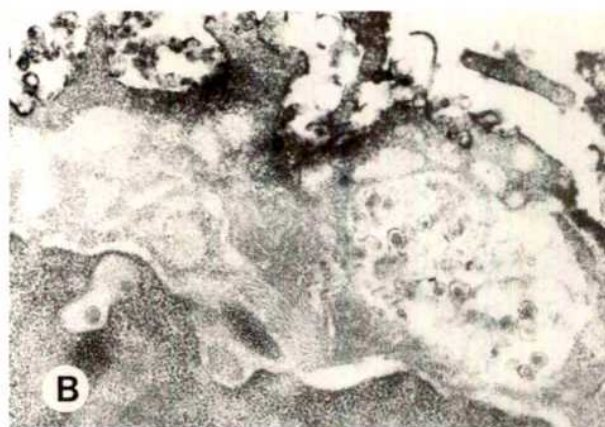
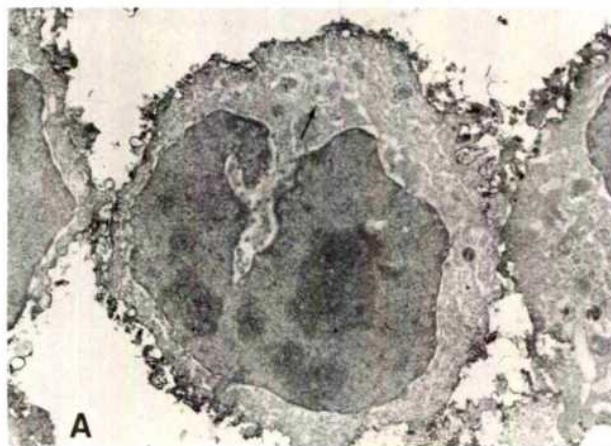


Fig 1. Immunoelectron micrograph of MT-2 cells. The plasma membrane is positively stained with the FTF 148 antibody. Virus particles in the vesicular structure are not stained (arrow). (A) Micrograph of MT-2 cells stained with uranyl acetate and lead citrate (original magnification $\times 7,300$). (B) Enlarged micrograph of MT-2 cells in which virus particles in a vacuole are visualized (original magnification $\times 31,200$).

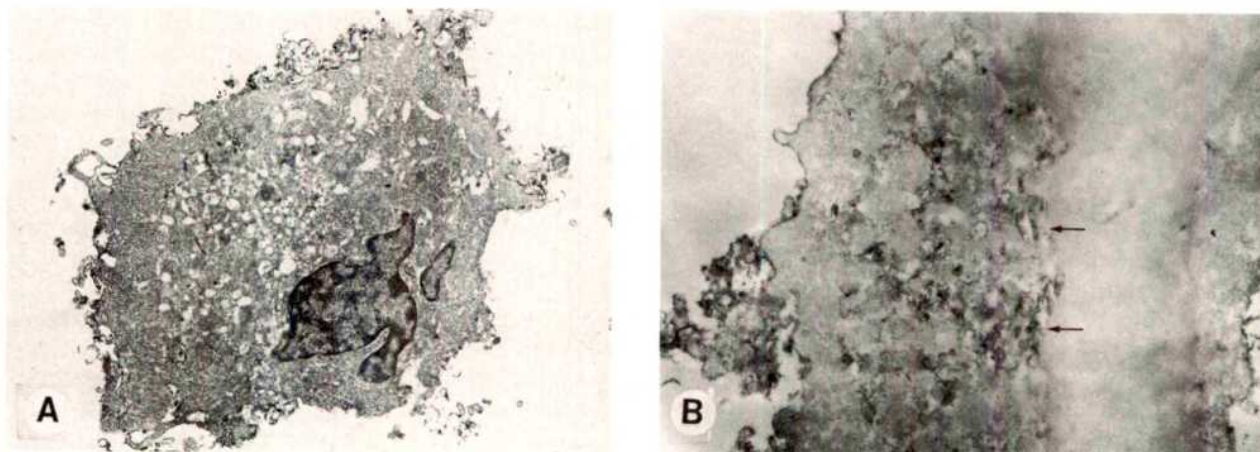


Fig 2. Immunoelectron micrograph of HUT-102 cells. In HUT-102 cells not only is the plasma membrane positive with the FTF 148 antibody, but the rough endoplasmic reticulum and some parts of the nuclear membrane are positively stained (arrow in B). (A) was stained with uranyl acetate and lead citrate (original magnification $\times 7,300$), but (B) was not stained (original magnification $\times 14,600$).

We prepared a new monoclonal antibody, FTF 148, and detected a novel antigen specifically expressed in HTLV-I-infected cells. This antigen is expressed in both T and B cell lines. According to our experience, the establishment of B cell lines from PBL of ATL patients is easier than that from PBL of normal persons. As demonstrated by Sugamura et al, HTLV-I infects B cell lines and induces the expression of IL 2 receptor molecules.²¹ This antigen is, however, different from the IL 2 receptor because the latter is also expressed in lectin-stimulated normal PBL but the former is not. Because the IL 2 receptor molecules of HTLV-I-infected T cells are, for the most part, low-affinity IL 2 receptor molecules, it was necessary to rule out the possibility

that the FTF 148 antibody reacted specifically with them. We used three monoclonal antibodies prepared and characterized by Tanaka et al¹⁴ to examine whether low-affinity IL 2 receptor molecules were well expressed in our lectin-stimulated PBL. The antibody specific to low-affinity IL 2 receptor molecules (H-A26) reacted with our lectin-stimulated PBL to the same extent as the antibody specific to high-affinity IL 2 receptor molecules (HIE1), and the antibody reacted with both high- and low-affinity molecules (H-31) by a membrane immunofluorescence method. The mol wt of this antigen and the IL 2 receptor is different. The anti-IL 2 monoclonal antibodies precipitated glycoproteins with mol wts of 60,000 to 65,000,²²⁻²⁴ whereas the FTF 148 antibody precipitated proteins with mol wts of 74,000 and 50,000. Taken together, the antigen presented in this paper is different from the IL 2 receptor. The possibility that the FTF

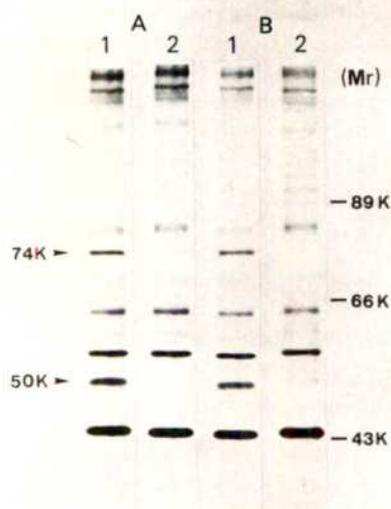


Fig 3. Immunoprecipitation and SDS-PAGE of ^3H -leucine-labeled KUT-2 cells. The cells were metabolically labeled with ^3H -leucine for 16 hours and subjected to immunoprecipitation with the FTF 148 antibody (A-1) or with normal mouse IgG (A-2). (B) KUT-2 cells were incubated with tunicamycin ($0.8 \mu\text{g}/\text{mL}$) for one hour, then ^3H -leucine was added, and the incubation was continued for 15 hours. The cells were subjected to immunoprecipitation with the FTF 148 antibody (B-1) or normal mouse IgG (B-2).

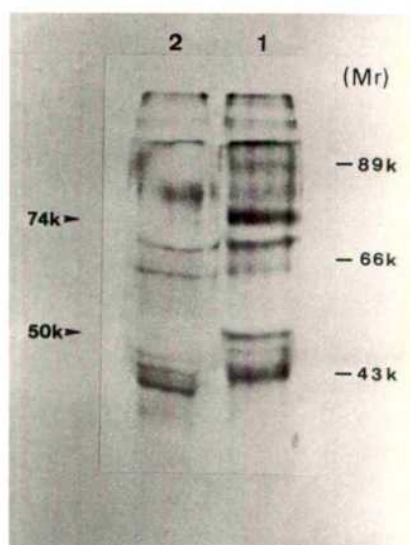


Fig 4. Immunoprecipitation and SDS-PAGE of ^{125}I -labeled KUT-2 cells. The cells were surface labeled with ^{125}I and subjected to immunoprecipitation with the FTF 148 antibody (1) or normal mouse IgG (2).

148 antibody is directed to an Ia-like antigen was ruled out because this antibody did not react with PBL from normal adults or with the B cell leukemia cell lines (B-ALL and B-CLL).

The antigen detected with the FTF 148 antibody is considered to be different from viral antigen because (1) this antibody reacted with the vast majority of MT-1 and KUT-2 cells, though the percentage of ATLA-positive cells in these cell lines was lower than 3%; (2) this antibody reacted with the ATL cells cultured for four hours although ATLA-positive cells were still very rare; (3) in MT-2 cells, many virus particles were found in the vesicular structure as well as in the extracellular space by electron microscopy, and these particles reacted with anti-ATLA-positive human serum and the rabbit antibodies raised against the highly purified virus solubilized with Triton X-100,²⁵ whereas, the FTF 148 antibody reacted only with the plasma membrane of MT-2 cells; and (4) HTLV-I-specific polypeptides exist in the cells in diverse forms.^{26,27} The *gag* gene products are synthesized as 53,000-dalton polypeptides and are proteolytically cleaved to three low-mol wt *gag* proteins (P19, P24, and P15). The *env* protein is synthesized as 46,000-dalton polypeptides and are glycosylated to 62,000-dalton molecules. These proteins are cleaved to 46,000- and 20,000-dalton molecules. After tunicamycin treatment of HTLV-I-infected cells, the 46,000-dalton polypeptides are not glycosylated, and 62,000-dalton molecules are not formed. These results strongly suggest that the antigen reactive with the FTF 148 antibody is different from viral proteins and their precursors.

The other genes that produce proteins are the *pol* and *Xs* genes. The *pol* gene protein of the retrovirus that is known as reverse transcriptase is in general synthesized as a *gag-pol* read-through product and is often detected as a 180,000- to 200,000-dalton polypeptide.^{28,29} The nucleotide sequence of HTLV-I suggested that the *pol* gene of this virus could code for a 99,000-dalton protein.⁶ Neither *gag-pol* product nor *pol* gene product has been identified yet in HTLV-I-infected cells, though the reverse transcriptase activity was clearly demonstrated on HTLV-I virion particles.^{5,30} One of the unique features of HTLV-I is its gene termed *Xs*, which resides downstream of the *env* gene and is flanked by 3'LTR. Recently, using rabbit antibodies to the synthetic oligopeptide specific to the predicted amino acid sequences of the product of the *Xs* region, 40,000-dalton proteins were detected in HTLV-I-infected cells.^{31,32} Previously Sugamura et al reported that a 40,000-dalton protein was detected with the serum of ATL patients in almost all the cell lines infected with HTLV-I but not in the uninfected cell lines.²⁰ We prepared a monoclonal antibody (MI 73) against the synthetic oligopeptide of the COOH terminal region of pX IV (the product of a fourth open reading frame of the *Xs* gene) that specifically reacted with this 40,000-dalton protein. By an immunofluorescence method, the 40,000-dalton protein was found to be distributed in the cytoplasm (polysome) and

nucleus of the major population of HUT-102, KUT-1, and KUT-2 cells in which the expression of ATLA was in a minor population (HUT-102, KUT-2) or not expressed at all (KUT-1) (unpublished observation). The pX I-III proteins have not been found yet, but this region may have no essential role for cellular transformation and may not function to synthesize proteins, as suggested by the low homology with HTLV-II. These results strongly suggest that the antigen we detected is different from the *pol* and *Xs* gene products.

We examined the reactivity of the FTF 148 antibody with fresh PBL from ATL patients. In some cases, many PBL reacted with antibody. In the majority of cases, however, only a low percentage of PBL reacted, or no PBL reacted. Franchini et al³³ have recently reported that in a few cases the messenger RNA transcribed from some part of the HTLV-I genome was expressed in fresh PBL obtained from ATL patients. We examined the lymph nodes of ATL patients by an immunohistochemical method with the result that in three patients out of four more than 10% of the lymph node cells expressed this antigen, thus indicating that in the tissue in which ATL cells are very actively proliferating (checked with Ki-67 monoclonal antibody) this antigen is expressed in a high percentage of ATL cells (unpublished observation). These results suggest that gene expression of HTLV-I induces the expression of this antigen. This antigen was expressed 1% to 2% of Raji cells that are not infected with HTLV-I. We presume that in Raji cells that have been cultured for many years, many cellular genes are newly expressed by mutations or chromosome translocations. This antigen has also expressed in the UW-4 cell line derived from an ATL patient and still depends on the presence of IL 2 for its proliferation. There is still a controversy as to whether ATL cells depend on IL 2 or not. The PBL from the ATL patient proliferate very well in the presence of IL 2, and from almost all cases IL 2-dependent permanent cell lines are established. This phenomenon is in high contrast to cultures of PBL from normal persons because the proliferation of the latter cells gradually decreases and it is very hard to establish a permanent cell line. This phenomenon suggests that ATL cells are immortalized by the integration of HTLV-I proviral DNA. From the IL 2-dependent cells derived from ATL lymphocytes, the conversion to IL 2-independent cells rarely occurs. At present we consider that there exist two different stages of ATL with respect to the dependence on IL 2. These results suggest that this antigen may be related to cell immortalization and is a product of certain cellular genes activated by some transacting factor (eg, Px) produced by HTLV-I infection.

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Spectrum of Natural Antibodies Against Five HTLV-III Antigens in Infected Individuals: Correlation of Antibody Prevalence With Clinical Status

By Genoveffa Franchini, Marjorie Robert-Guroff, Anna Aldovini, Nancy C. Kan, and Flossie Wong-Staal

The genome of the HTLV-III/LAV retrovirus, the etiologic agent of the acquired immunodeficiency syndrome (AIDS), encodes the viral structural proteins (envelope and core proteins), the reverse transcriptase, a transactivation protein (tat-III), as well as two other proteins (3'orf, sor) of unknown function. We studied the prevalence of natural antibodies against envelope, gag, 3'orf, sor, and tat-III in the sera of HTLV-III infected individuals in an attempt to correlate clinical status with seropositivity to specific HTLV-III antigens. We selected 101 sera; 16 were obtained from normal donors with no known risk factors, and 85 were from patients with full-fledged AIDS (28 cases), AIDS-related complex (ARC, 22 cases), and healthy people at risk (homosexuals, intravenous [IV] drug users, relatives of AIDS patients; 35 cases). Seropositivity for antibodies against the envelope (gp41) and gag antigens (p15, p24) was determined by Western blot using disrupted HTLV-III virions. Of the 101 sera, all 16 from nonrisk donors and 3/35 from healthy at-risk donors were negative for antibodies against either the gp41 or p15 and p24. The remaining 82 sera were seropositive for either the gp41

and/or the p15 and p24. All sera were then tested against the three known HTLV-III antigens (3'orf, sor, and tat-III) that have been synthesized in bacteria. Our data indicate that all the HTLV-III antigens tested are immunogenic in vivo. No significant difference in antibody prevalence to gp41 (close to 100%) and to the 3'orf, sor, and tat-III proteins (approximately 50%) was observed with regard to stage of the disease. In contrast, the prevalence of antibodies against the core antigens decreased from approximately 100% in infected people with no clinical signs of disease to 50% in ARC and AIDS patients. The percentage of patients seropositive for all five antigens tested was increased in the AIDS group. These results indicate that the greatest antibody prevalence was obtained using viral envelope antigen and further suggest that screening with the newly identified 3'orf, sor, and tat-III proteins as antigens would confer no further diagnostic advantage. The pattern of natural antibodies observed during disease progression did not suggest any pathogenetic mechanism. © 1987 by Grune & Stratton, Inc.

ACQUIRED immunodeficiency syndrome (AIDS) was first described in the United States in 1981,¹ and since then the disease has reached epidemic proportions.² The syndrome consists of a series of clinical manifestations directly related to the severe immune dysfunction in the affected people, such as opportunistic infections and various disorders such as autoimmune diseases, malignancies, and neurologic manifestations, the pathogenetic origin of which is less obvious. Infection by HTLV-III/LAV is the common denominator among these patients.³⁻⁵ HTLV-III infection correlates with depletion of the OKT⁴⁺ T cells in vivo and in vitro.^{6,7} While the immunodepression observed in patients with AIDS could be a direct consequence of the cytopathic effect of HTLV-III exerted on T cells as it is observed in vitro,⁷ the role played by HTLV-III in the pathogenesis of the various clinical manifestations of the syndrome is still not clear. The molecular cloning and the nucleotide sequencing of HTLV-III^{8,9} allowed the identification of the envelope, polymerase and the core protein genes, and three additional open-reading frames, 3'orf, sor, and tat-III, that encode proteins of 27, 23,¹⁰⁻¹² and 14Kd respectively.¹³ The tat-III protein seems to enhance virus expression in the infected^{14,15} cells and may play a key role in the mechanism of viral replication. The functions of the 3'orf and sor proteins relative to the viral life cycle or the cellular metabolism of infected cells remain to be uncovered. We studied the expression in vivo of these viral genes by analyzing the natural antibody response in infected individuals in an attempt to correlate the stage of the disease and the different clinical manifestations with the expression of specific viral proteins.

MATERIALS AND METHODS

Virus preparation. HTLV-III produced by H9 cells was harvested from 50 liters of cell-free tissue culture media using zonal ultracentrifugation on two sequential ribonuclease-free sucrose gra-

dients as previously described.¹⁶ Purified virus was disrupted in 50 mmol/L Tris-HCl buffer, pH 7.9, containing 0.25% Triton X-100, 20 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.5 mol/L KCl, and 1 mmol/L phenylmethyl sulfonyl fluoride by stirring for one hour at 4 °C. The solubilized virus preparation was dialyzed extensively against phosphate-buffered saline (PBS) and centrifuged for one hour at 100,000 × g. The supernatant fraction was used in Western blot¹⁷ assays following protein estimation by the method of Lowry.¹⁸

Purification of the bacterially synthesized viral proteins. The bacterially synthesized HTLV-III proteins were purified by different methods. The 3'orf protein was obtained from the total cell lysate of E 10 bacteria after solubilization in 50 mmol/L Tris-HCl, pH 8, containing 1.5 mol/L sodium chloride and 8 mol/L urea and application of the extract to a Sephacryl S-300 column. The fractions containing the 3'orf protein were pooled and applied to a Sepharose 4B column coupled with an antibody directed against the amino terminus of the hybrid protein.¹⁰ The 3'orf protein was recovered by elution with 2% formic acid and used in a Western blot assay.¹⁷ The bacterially synthesized 27 Kd 3'orf protein is a hybrid protein that comprises 84% of the 3'orf gene and 36 amino acids derived from the prokaryotic vector.¹⁰ By coincidence the molecular weight of the bacterial protein is the same as the molecular weight of the native protein (p27) detected in infected cells.^{10,11} The sor protein was purified by application of the bacterial lysate¹² to a cation-

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exchange Water Protein-Pak Sp-5PW HPLC column and elution with a 25-mL gradient of 0% to 80% 0.75 mol/L NaCl in the equilibration buffer. The bacterial *sor* (p18) is a hybrid protein representing 83% of the *sor* gene. The native protein product has been identified as a 23Kd protein.¹² The tat-III protein was purified¹³ by applying the bacterial lysate to a 15% SDS-polyacrylamide gel. After electrophoresis the 14 Kd protein was eluted from the appropriate gel slice. The 14 Kd tat-III protein expressed in *Escherichia coli* is not a fusion protein but represents the complete protein product of the tat-III gene. It therefore has the same size as the native protein product detected in infected cells.¹³

Western blot analysis of the viral proteins. Lysate of HTLV-III or purified recombinant proteins (3'orf, tat-III, and *sor*) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred to nitrocellulose sheets as described,¹⁷ and the sheets were incubated for one hour at room temperature with a solution containing 5% nonfat dry milk and 0.01% Antifoam (Sigma, St Louis) in PBS to block nonspecific protein-binding sites. Strips were obtained from the nitrocellulose sheets and individually reacted with human sera diluted 1:100 for one hour at 4°C in the same solution containing 5% nonfat dry milk. After incubation the strips were washed in PBS containing 0.5% sodium deoxycholate and reacted for 20 minutes with ¹²⁵I staphylococcal protein A (5 × 10⁵ cpm/mL). The strips were washed again, and autoradiograms were obtained.

RESULTS

Location of the coding regions for the HTLV-III proteins. A summary of the genes identified in the different open-reading frames of the HTLV-III genome by computer analysis is depicted in the upper part of Fig 1. To date the core proteins p24, p15, have been identified as well as the gp120 and gp41^{3,19,20} for the exterior and transmembrane portion of the envelope respectively. Three other HTLV-III genes have been uncovered by studying the viral c-DNAs,¹⁴ and the features of their spliced mRNAs are summarized in the lower part of Fig 1. The native products of the three genes, 3'orf, *sor*, and tat-III have been identified in infected cells cultured in vitro. The three genes encode proteins p27,¹⁰⁻¹¹ p23,¹² and p14¹³ respectively.

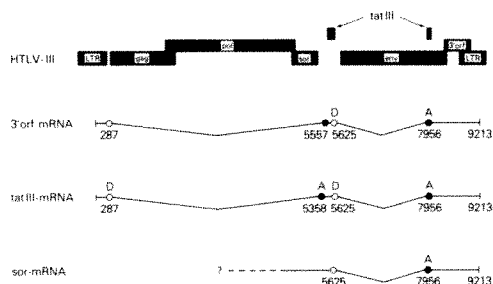


Fig 1. Diagrammatic representation of the HTLV-III genome and of the spliced mRNAs for three protein products. The upper part of Fig 1 displays the HTLV-III genome and the position of the open-reading frames for the six genes that have been identified. The schemes underneath the HTLV-III map include the sequences that are maintained in the mature mRNAs for the 3'orf, *sor*, and tat-III proteins. The white and black dots represent the donor and acceptor sites that are joined together in the mature mRNA. The 5' donor splice site for the *sor* mRNA has not been identified yet. The numbers refer to the position of the sites in the HTLV-III nucleotide sequence.⁹

Correlation of antibody response and stage of the disease. We attempted to correlate the stage of the disease with the prevalence of antibodies against specific viral antigens. We employed 82 human sera seropositive for antibodies against the HTLV-III core proteins (p15 and/or p24) or the gp41 or both. The sera, all obtained from Americans, included 32 samples from healthy individuals at risk for HTLV-III infection; 28 samples from patients with full-fledged AIDS, and 22 samples from American patients with ARC. The patients in the risk group defined 'healthy' included individuals with and without lymphadenopathy (LAS). Among them, three were IV drug users and one was a relative of a patient with AIDS. The rest were homosexuals. ARC patients were staged according to the criteria of the Centers for Disease Control,²⁰ and the group included patients with at least two laboratory findings of immunodysfunction and two clinical manifestations of disease. All the AIDS patients had serious manifestations of disease, including either Kaposi's sarcoma (50%) or opportunistic infections (50%). We also studied 16 human sera seronegative for antibodies against the *gag* and envelope proteins of HTLV-III obtained from a population not at risk for HTLV-III infection and three sera from previously identified virus-positive, antibody-negative healthy carriers.^{21,22} An example of a Western blot of some of these sera using disrupted HTLV-III is given in Fig 2, first panel. The sera were also reacted against the purified 3'orf, *sor*, and tat-III protein products expressed in *E coli* (Fig 2, panels 2, 3, and 4). Some sera from each group of patients (healthy at risk, ARC, AIDS) recognized the bacterially synthesized HTLV-III proteins. Sera of the normal donors (HN) lacking antibodies to the envelope and the core proteins of HTLV-III also did not react with the tat-III and the 3'orf proteins; in contrast some of the normal sera recognized the *sor* protein. The three sera obtained from the healthy virus-positive individuals failed to recognize the 3'orf, *sor*, and tat-III proteins. Later samples from these same individuals seroconverted and became positive for envelope and core antigens.

A histogram of the distribution of natural antibodies against the five HTLV-III proteins analyzed in this study is shown in Fig 3. The antibody prevalence against the envelope antigen gp41 was constant in all phases of the disease. Almost all patients tested were seropositive for this antigen, in agreement with other studies²³ that have shown the viral envelope to be the major target antigen in HTLV-III-exposed individuals. While minor fluctuations in antibody prevalence were observed with disease progression, overall a constant pattern occurred with the 3'orf, *sor*, and tat-III proteins. In the case of these antigens, however, the percentage of seropositive individuals was in the intermediate range of 30% to 65% depending on the antigen. The antibody prevalence to the *gag* antigenic determinants was greatest among the healthy at risk group, as reported also by other investigators.²⁵ When individuals in the early phases of disease were analyzed with regard to the presence or absence of lymphadenopathy, no differences in the antibody prevalence to any of the five antigens were detected.

Specificity of seroreactivity to the bacterially synthesized *sor* protein. As indicated in the histogram of Fig 3, 25% of

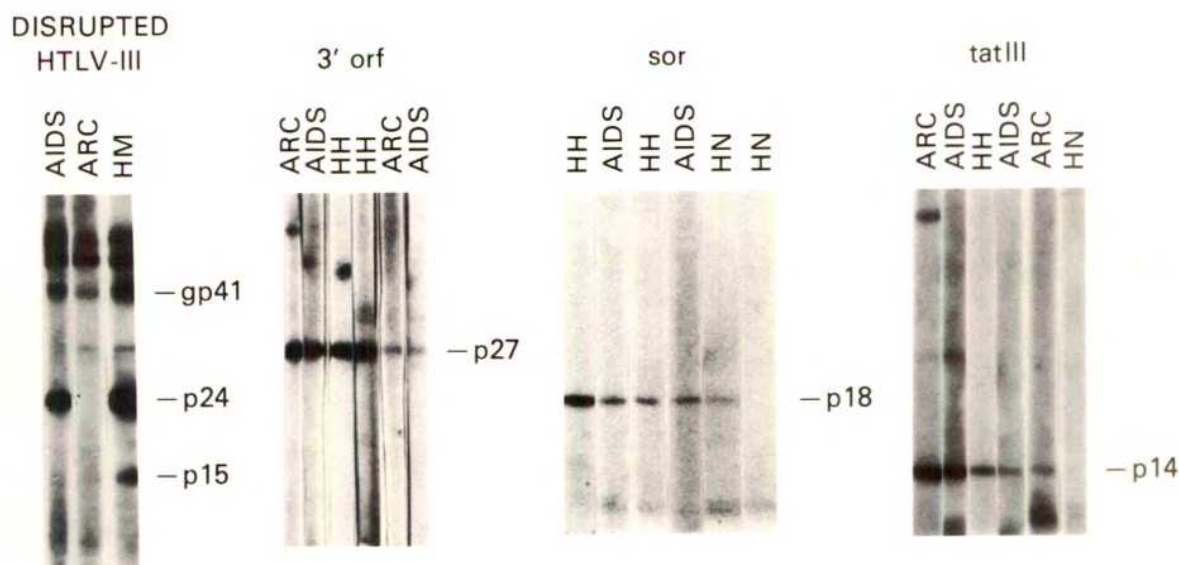


Fig 2. The first panel represents the autoradiograph of nitrocellulose strips containing HTLV-III viral proteins from the disrupted virion reacted with various human sera. Panels 2, 3, and 4 represent examples of the reactivity of human sera toward the 3' orf, sor, and tat-III purified proteins respectively. The molecular weight of the proteins was derived from molecular weight standard proteins electrophoresed on the same gel (Bethesda Research Laboratory, Bethesda, MD). HN, HH, and HM refer to human sera and represent a healthy, normal, not-at-risk patient; healthy homosexuals; and a healthy mother of a child with AIDS. AIDS and ARC stand for sera obtained from patients with the AIDS syndrome and from patients with AIDS-related complex.

sera from uninfected individuals reacted with the recombinant 18Kd sor protein. This reactivity was specific for the recombinant protein, since a protein of similar size is not detected in the control bacterial lysate (Fig 4, lanes 1 and 2). Similarly, the specific antiserum obtained from a rabbit immunized with the purified protein reacted with the recombinant sor protein (lane 4), while no reaction was observed in the total bacterial lysate (lane 3). The sor protein used in these studies is a chimeric protein that contains at the amino terminus 13 amino acids derived from the ω II gene of the vector.¹² To rule out that the reactivity of the human sera was directed against vector epitopes rather than sor epitopes, we tested the reactivity of the same sera against another chimeric protein expressed in the same vector containing 27% of the coding sequences of the HTLV type I envelope gene p21E.²⁴ Figure 4, lanes 5 and 6, shows that a human serum positive for the recombinant sor protein does not recognize the p21 E recombinant HTLV-I chimeric protein, while a

serum obtained from a patient with adult T cell leukemia does. Thus, the reactivity of human sera from uninfected individuals seems to be directed against the antigenic determinants of the recombinant sor protein.

DISCUSSION

All the known proteins encoded by HTLV-III are immunogenic in vivo, but the spectrum of antibodies that they

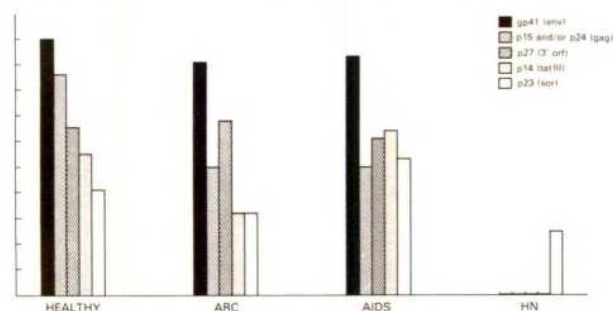


Fig 3. The spectrum of natural antibodies in patients' sera is expressed as a percentage of total patients analyzed for each antigen, which is represented on the Y axis. The legend for each antigen is reported in the right upper part of the figure.

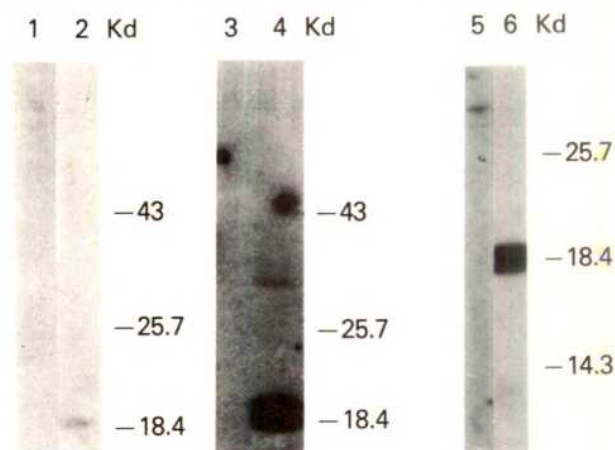


Fig 4. Total cell lysate from bacteria expressing the sor protein (lanes 2 and 4) and from control bacteria (lanes 1 and 3) were analyzed by Western blot using a human serum from an uninfected individual (lanes 1 and 2) and a rabbit antiserum raised against the recombinant sor protein (lanes 3 and 4). A Western blot against the purified recombinant protein containing the antigenic determinants of the HTLV-I p21E (lanes 5 and 6) was obtained with the same human serum used in lanes 1 and 2 (lane 5) and with a serum from a patient with adult T cell leukemia (lane 6).

elicit in infected individuals does not seem to correlate with disease stages as they are currently described. We noticed that as infected individuals progressed toward AIDS, they tended to acquire antibodies to all the HTLV-III antigens studied (data not shown). This finding may simply reflect continual expression of the virus in vivo with a greater likelihood of exposure to each antigen with increasing time after infection. Fluctuations in antibody prevalence were observed with stage of disease, particularly with the *sor* and *tat*-III proteins, although the differences observed did not yield statistically significant correlations. These varying prevalences may result from different levels of circulating antigens with disease progression and immune complex formation, leading to lessened detection of serum antibodies. More extensive studies would be necessary to determine whether the observed fluctuations have underlying biologic significance. Direct analysis for circulating immune complexes or complexes trapped in lymph nodes would be informative. It is remarkable that the immune response against all HTLV-III antigens in AIDS patients seems to be comparable to that of the healthy at-risk patients, despite the high degree of immune system failure observed in the former group. Our data suggest that it may be necessary to investigate individuals very soon after viral infection to determine whether sequential exposure to HTLV-III antigens is correlated with laboratory or clinical manifestations of AIDS. In this regard we analyzed sera of three patients at risk who were virus positive, seronegative for antibodies against the HTLV-III envelope and *gag* proteins and who subsequently seroconverted. Their sera were negative for the 3'orf, *sor*,

and *tat*-III before the time of seroconversion, suggesting that these proteins might not be useful in the diagnosis of the early phase of infection. More serum samples from this kind of patient should be studied before a definitive conclusion on this point can be reached. Our analysis has shown that of the five HTLV-III antigens studied, the most immunogenic one appears to be the gp41 transmembrane protein. As the major envelope protein gp120 also appears to be highly immunogenic,²³ it would be useful to include it in screening procedures when the purified protein becomes available. Obviously the use of a combination of bacterially synthesized antigens for large scale antibody testing not only could increase the sensitivity of antibody detection but could also eliminate the problem of obtaining false-positive results due to contamination of virus preparations with human antigens. In this regard it should be noticed that the *sor* protein should not be used in the antibody screening procedure because it could be the cause of false-positive results. In fact, we have shown that some human sera from not-at-risk, uninfected individuals have antibodies that react with the *sor* antigenic determinants. We believe that this reactivity may be due to crossreactive determinants in either endogenous or exogenous proteins that elicit an immune response in humans.

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Formation and Functioning of the Factor IXa-VIII Complex on the Surface of Endothelial Cells

By Katalin Váradi and Susan Elödi

The formation and functioning of the factor X activating complex on the surface of cultured human venous endothelial cells (HVEC) were investigated. To the HVEC monolayer human factors IXa, VIII, X, CaCl_2 , and S-2222 were added, and a gradually increasing activation of factor X was observed. The maximum activity of 88 nmol/L Xa/min was reached after a 12-minute lag phase. In the presence of thrombin-activated factor VIII (VIII_t) the same maximum activity developed in eight minutes, which suggests that VIII_t accelerates the formation of the IXa-VIII complex but does not influence its factor X-activating potential. Anti-VIII IgG did not affect the activity of the full-fledged

complex. When anti-VIII IgG was added to the reaction mixture before factor VIII or during the lag phase of the reaction, it induced a concentration-dependent decrease of factor X activation. These results indicate that endothelial cells provide a binding surface for the IXa-VIII complex and that in the HVEC-bound complex factor VIII is protected from the effect of a specific antibody. However, the relatively slow development of the maximum activity indicates that HVEC only partially satisfy the surface criteria for the optimal assembly of the IXa-VIII complex.

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EVENTS LEADING TO THROMBUS formation are thought to be initiated by damage to the vascular endothelium. The intact endothelial cell lining is generally believed to provide a nonthrombogenic surface for blood.¹ Recently, it became evident that the endothelium cannot be regarded as an inert lining but that the endothelial cells actively contribute to the maintenance of blood fluidity. Endothelial cells synthesize and release prostacyclin,² which inhibits platelet aggregation, as well as plasminogen activator,³ which contributes to the dissolution of fibrin.

Thrombomodulin, an endothelial cell membrane protein, binds thrombin and protein C with high affinity.^{4,5} Protein C activated by thrombin effectively inactivates factors V and VIII⁶; thus it serves as a regulator of coagulation. In contrast to these nonthrombogenic functions, recent data suggest that reactions promoting clot formation may also take place on the surface of intact endothelial cells. Both zymogen and active factors IX and X can attach to the surface of vascular endothelial cells.⁷⁻⁹ Bovine aortic endothelial cells synthesize factor V. In the presence of exogenous factor X and calcium, the formation of the prothrombinase complex and production of thrombin was demonstrated on the surface of bovine aortic endothelial cells.¹⁰

Previously we found that the complex of factor IXa and factor VIII is formed on the surface of activated platelets.¹¹ Complex-bound factors IXa and VIII display novel properties of being resistant to the effect of specific inhibitors.¹²

The aim of this work was to investigate whether endothelial cells could provide an appropriate surface for the formation and functioning of the factor IXa-VIII complex. We observed that, albeit slowly, the IXa-VIII complex was formed and it activated factor X in the presence of intact endothelial cells. In the endothelial cell-bound complex, factor VIII became resistant to the effect of anti-VIII

antibody, similarly to that observed when the complex was formed on the surface of aggregated platelets.

MATERIALS AND METHODS

Coagulation proteins. All clotting factors used in these experiments were prepared from normal human blood. Factor IXa (194 U/mg) was prepared as described earlier.¹³ Factor VIII (26 U/mg) was purified from a therapeutic factor VIII concentrate by Sepharose 4B chromatography according to Van Mourik and Mochtar.¹⁴ Factor X (6 U/mg) was isolated from a prothrombin complex concentrate by dextran-sulphate-Sepharose chromatography.¹⁵ Anti-VIII IgG (210 BU/mg) was prepared from the plasma of a nonhemophilic patient who developed antibody against factor VIII. The IgG₄ fraction was obtained by QAE-Sephadex chromatography¹⁶ and was further purified by the caprylic acid method of Steinbuch and Audran.¹⁷

Human venous endothelial cells. Isolated from the umbilical cord vein, human venous endothelial cells (HVEC) were cultured as described by Jaffe.¹⁸ During the second passage the cells were plated in culture wells with a 16-mm diameter and were allowed to grow to confluence for 24 hours. One well contained approximately 10⁵ cells. HVEC were cultured and kindly provided by the Department of Transplantation Immunology of our institute.

Assay of clotting factors. Factor IXa activity was measured by a one-stage clotting assay as described previously.¹⁹ The activity of factor VIII was measured by a one-stage assay on factor VIII-deficient human plasma and was related to a freeze-dried local plasma standard calibrated for the Second International Standard for factor VIII (73/552 National Institute for Biological Standards and Control, London). The activity of factor Xa was measured with the chromogenic peptide substrate Bz-Ile-Gly-Arg-pNA (S-2222, KABI Diagnostica, Stockholm) and was related to the factor X plasma standard of Immuno AG (Vienna). Russell's viper venom (RVV, Stypven, Burroughs Wellcome Ltd, Beckenham, England) was used for the activation of factor X when preparing the calibration curve for factor Xa assay. The factor VIII inhibitory capacity of the anti-VIII IgG preparation was measured by the Bethesda method and expressed in Bethesda units.²⁰

Protein assay. The protein content of the clotting factor preparations was determined spectrophotometrically by using the absorption coefficients $A_{280}^{1\%}$: 12.3 for factor VIII,²¹ 13.3 for factor IXa,²² and 12.4 for factor X.²³ The protein content of the anti-VIII IgG preparation was measured according to Lowry et al²⁴ by using bovine serum albumin (Miles Laboratory, Elkhart, IN) as a reference.

Experiments with endothelial cells. Confluent cells from the second passage were washed three times with imidazole-buffered saline (IBS), pH 7.2, composed of 5 mmol/L imidazole HCl and 140 mmol/L NaCl. Then, factor IXa (15.5 μg), factor VIII (1.1 units),

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factor X (90 μg), 10 mmol/L CaCl_2 , and 1.3 mmol/L S-2222 in IBS were added in a final volume of 750 μL . The assay mixtures were incubated at 37 $^\circ\text{C}$ and tilted at 60-second intervals for mixing. Fifty-microliter aliquots were withdrawn at different time intervals and diluted with ice-cold IBS containing 10% acetic acid to terminate the reaction.

The activation of factor X was followed by measuring the difference in the absorption of the samples at 405 nm. The amount of factor Xa formed was read from a calibration curve obtained by measuring the activation of 50 to 500 nmol/L factor X in the presence of 10 $\mu\text{g}/\text{mL}$ RVV, 40 mmol/L CaCl_2 and 1.3 mmol/L S-2222.

When thrombin-activated factor VIII (VIII_t) was used in the experiments, factor VIII was preincubated at 37 $^\circ\text{C}$ with 0.05 NIH units/mL thrombin (Topostasine, Hoffman-LaRoche, Basel, Switzerland). A tenfold increase in factor VIII activity was reached in one minute, and then an aliquot was added to the reaction mixture in a final concentration of 1 U/mL.

The HVEC-bound reaction was compared with that taking place in the presence of phospholipid (PL). In these experiments, 10 μg PL (Inosithin, Associated Concentrates, Long Island, NY) was added to the reaction mixture instead of HVEC. The amount of PL was optimized for the concentration of clotting factors as described previously.²⁵

Calculations. The best fitting curves for the experimental points and the appropriate time derivatives were calculated and drawn by a microcomputer applying a polynomial regression analysis program.

RESULTS

Time course of factor X activation. The activation of factor X in the presence of confluent HVEC under various conditions is shown in Fig 1. When all components required for the activation of factor X were added to HVEC (Fig 1A, curve a) after a three- to four-minute lag phase, the increase in absorbancy indicated that the activation of factor X had been initiated. After 12 minutes' incubation, the change in absorbancy became proportional to time, thus suggesting

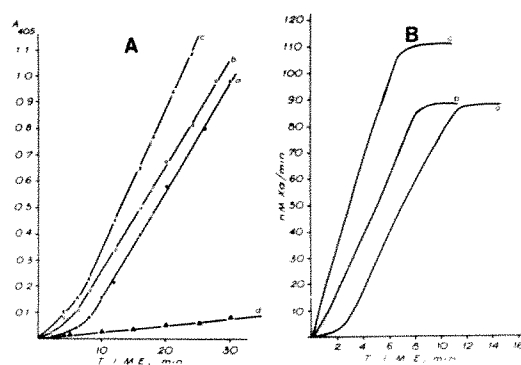


Fig 1. Time course of activation of factor X by the IXa-VIII complex. (A) The activation of factor X was monitored by the increase of absorbancy due to splitting of S-2222 by factor Xa formed in the assay system. Factor Xa was generated in the presence of HVEC, Ca, IXa, and VIII (curve a); HVEC, Ca, IXa and VIII, (curve b); PL, Ca, IXa, and VIII (curve c); and HVEC, Ca, and IXa (curve d). (B) Time derivatives of the experimental curves of Fig 1A showing the kinetics of factor X activation during the assembly of the IXa-VIII complex. Symbols are the same as in (A). For details of the experiments and calculations see Materials and Methods.

that the activation of factor X reached its maximum velocity. When factor VIII was omitted from the reaction mixture (Fig 1A, curve d), factor IXa activated factor X only at a low rate. To exclude the possibility of any nonspecific binding of the investigated factors to the culture wells, all components needed for factor X activation were incubated in empty wells, but in this case activation of factor X was not detected (not shown).

Previously we found that VIII_t in the presence of PL or platelets accelerates the formation of the IXa-VIII complex.^{11,25} Curve b in Fig 1A illustrates that a similar phenomenon occurs when the IXa-VIII complex is formed in the presence of HVEC. The addition of VIII_t to the reaction mixture induced a more rapid increase in absorbancy already at the beginning of the reaction, thus indicating the accelerating effect of VIII_t . However, even with VIII_t , the rate of activation of factor X was less in the presence of HVEC than that measured in the control experiment with PL and factor VIII without thrombin pretreatment (curve c, Fig 1A).

It was assumed that the maximum velocity of factor X activation is reached after the assembly of factors IXa and VIII in the complex. The kinetics of the formation of the IXa-VIII complex are shown in Fig 1B where the velocity of factor X activation in the course of the assembly of the IXa-VIII complex is illustrated. When the complex is formed with untreated factor VIII (Fig 1B, curve a) the maximum activity, ie, the production of 88 nmol/L Xa/min, is reached after 12 minutes' incubation, whereas with VIII_t (Fig 1B, curve b) the maximum activity is measured after eight minutes. The accelerating effect of VIII_t is most pronounced at the beginning of the reaction where a steady increase in the reaction rate is seen. In contrast to this, in the presence of untreated factor VIII, significant activation of factor X does not occur for two minutes. Control experiments carried out with PL suggest that under these conditions PL provides a more favorable surface for the formation of the IXa-VIII complex than do HVEC. With PL preparation, the maximum activity, ie, the production of 110 nmol/L Xa/min, is reached after six minutes (Fig 1B, curve c).

It has to be pointed out that, although VIII_t increased the rate of formation of the IXa-VIII complex, it did not influence the maximum activity of the fully fledged complex. Irrespective of the state of factor VIII, the HVEC-bound complex produced 88 nmol/L Xa/min, ie, about 80% of that measured in the presence of PL.

Effect of anti-VIII IgG on HVEC-bound factor VIII.

The anti-VIII IgG used in our experiments inactivated factor VIII in solution as shown in Fig 2. When the antibody was added to the HVEC-bound complex after development of the maximum activity, the antibody appeared to be ineffective. This phenomenon suggests that the antigen determinants of the complex-bound factor VIII become masked and are not accessible for the specific antibody.

The marked differences observed between antibody-induced inactivation of free and complex-bound factor VIII raised the question whether the complex of factors IXa and VIII could be formed in the presence of anti-VIII IgG. As shown in Fig 3, when the antibody was added to the preincubation system at zero time, ie, at the beginning of

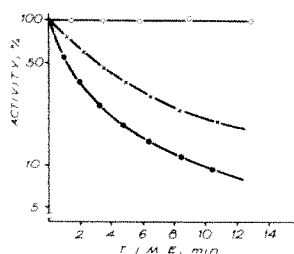


Fig 2. Effect of anti-VIII IgG on the activity of factor VIII and on the HVEC-bound IXa-VIII complex. Change of activity of 1 U/mL factor VIII at 37 °C in the presence of 5 BU/mL (x—x) and of 10 BU/mL (●—●) anti-VIII:C IgG. The activity of the HVEC-bound complex is also shown in the presence of 10 BU/mL anti-VIII IgG (○—○) added when the maximum activity of the IXa-VIII complex developed.

complex formation, the initial rate of factor X activation and the maximum activity of the complex decreased in proportion to the antibody concentration. When the antibody was added to the reaction mixture in the fifth minute of incubation, the inhibitory effect was less pronounced; 60% of the maximum activity developed even after adding 10 BU/mL antibody (Table 1). These data suggest that in the fifth minute of complex formation a part of factor VIII present is already incorporated into the complex and only the free factor VIII is still accessible for the antibody.

DISCUSSION

It is generally accepted that some reactions of the clotting process take place on the membrane of aggregated platelets. Two consecutive enzyme complexes of coagulation, ie, the IXa-VIII and the prothrombinase complex, have been shown to be formed on the membrane of platelets.^{11,26,27} Recent investigations, however, indicate that the platelets are not the only cells that can provide a binding surface for the clotting factors. Bovine factors IX and X bind to bovine aortic endothelial cells with high affinity.⁷ The endothelial cell-bound factor IX can be readily activated by factors XIa and VIIa.⁹ In the case of comparable cell numbers, the prothrom-

Table 1. Maximum Activity of the IXa-VIII Complex Developed in the Presence of Anti-VIII Antibody

Time of IgG Addition (min)	nmol/L Xa/min in the Presence of Antibody	
	5 BU/mL	10 BU/mL
0	40	21
5	60	49
11	88	88

binase complex was found to be more efficient on the surface of bovine aortic endothelial cells than in the presence of activated thrombocytes.¹⁰ However, HVEC appear to be less convenient for the binding and functioning of clotting factors than the bovine aortic endothelial cells. The prothrombinase complex, for example, produces thrombin at a considerably lower rate on human venous cells than on bovine aortic cells.¹⁰

Our experiments with HVEC show that in the absence of factor VIII factor IXa produces only 5 nmol/L Xa/min. When factor VIII was present, factor IXa produced 88 nmol/L Xa/min after a ten- to 12-minute lag phase, ie, factor VIII increased the activity of factor IXa about 18-fold. However, the maximum activity of the HVEC-bound IXa-VIII complex does not reach the value of 110 nmol/L Xa/min measured in the presence of PL in control experiments.

Our previous studies indicated that the rate-limiting step in the assembly of the IXa-VIII complex is the binding velocity of factor VIII.²⁸ The present data, however, suggest that the properties of the binding surface also have a marked influence on the rate of assembly of the complex. In the presence of HVEC, ten to 12 minutes was needed for the development of the maximum activity. Although VIII_i shortened this time to eight to nine minutes, this rate of assembly is still considerably slower than that measured previously with platelets and VIII_i, where the maximum activity developed in one minute.¹¹

Previously we found that in the presence of platelets complex-bound factor VIII had become protected from the effect of the specific anti-VIII antibody.¹² A similar protection was described in the case of PL-bound factor VIII.²⁹

Our experiments with HVEC demonstrate that the protection of the surface-bound VIII prevails in the presence of these cells too. In the experiments carried out with platelets, the presence of 2 BU/mL anti-VIII IgG prevented the formation of the IXa-VIII complex.¹² In contrast to this, when the formation of the IXa-VIII complex was investigated on HVEC, 25% and 50% of the maximum activity developed in the presence of 10 and 5 BU/mL anti-VIII IgG, respectively. These results suggest that in spite of the relatively slow assembly of the complex the HVEC offers a more effective protection for factor VIII than the platelet surface.

Although our results and the data of Rodgers and Shuman¹⁰ demonstrate that both the IXa-VIII and the prothrombinase complex are formed in vitro on the surface of endothelial cells, the question arises whether HVEC-bound complexes play a role in physiological coagulation. The rate

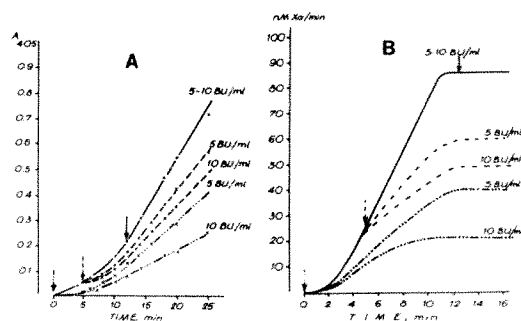


Fig 3. Formation of the HVEC-bound IXa-VIII complex in the presence of anti-VIII IgG. Five and 10 BU/mL anti-VIII IgG were added (arrows) to the reaction mixture at 12 minutes (—) or five minutes (---) of incubation and at time 0 (· · · · ·), ie, before the addition of factor VIII. (A) best-fitting curves of three experiments. (B) Time derivatives of experimental curves of (A).

of the assembly of the IXa-VIII complex is considerably slower on HVEC than that we have observed previously on the surface of platelets.¹¹ This indicates that in physiological coagulation platelets probably play a predominant role in providing binding surface for the IXa-VIII complex. If a

conclusion may be drawn for the in vivo processes from the data obtained with cultured cells, then it may be assumed that in the venous circulation the slow formation of the IXa-VIII complex on the surface of intact endothelial cells may contribute to the development of venous thrombosis.

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Monoclonal Antibodies to Discrete Regions in α_2 -Plasmin Inhibitor

By Jun Mimuro, Yukiya Koike, Yoshihiko Sumi, and Nobuo Aoki

Three monoclonal antibodies to α_2 -plasmin inhibitor (α_2 PI) were characterized. The first, JTPI-1, was directed against the reactive site of α_2 PI and inhibited antiplasmin activity by interfering with the formation of α_2 PI-plasmin complexes. The avidity of JTPI-1 to the preformed α_2 PI-plasmin complex was markedly lower than that to free α_2 PI, which made this antibody useful for measuring the free α_2 PI in plasma. The second, JTPI-2, recognized an epitope in the C-terminal fragment of α_2 PI (11,000 daltons [11 K]) that was cleaved from α_2 PI by plasmin upon complex formation but remained noncovalently attached to the complex. However, binding of JTPI-2 to α_2 PI was not inhibited by the C-terminal 26-residue peptide containing the plasminogen-

binding site and had no effect on the function of α_2 PI. These data suggested that JTPI-2 recognized an epitope between the C-terminal 26-residue peptide and the reactive site. The third, JTPI-3, bound the α_2 PI-plasmin complex (150 K) as well as α_2 PI. Binding was inhibited by the N-terminal 12-residue peptide of α_2 PI, but factor XIII-catalyzed cross-linking of α_2 PI to fibrin was not inhibited by JTPI-3. These results suggested that the antibody recognized an epitope near the N terminus. These three monoclonal antibodies were useful for analyzing the mechanism of interaction between α_2 PI and plasmin.

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THE α_2 PLASMIN INHIBITOR (α_2 PI) is a physiological inhibitor of plasmin¹⁻³ so that it acts as a major inhibitor of fibrinolysis in plasma.^{4,5} It inhibits a physiologically occurring fibrinolytic process that follows fibrin formation, thereby stabilizing hemostatic plugs.⁶ Its congenital deficiency results in a lifelong severe hemorrhagic tendency.⁷ The α_2 PI molecule has three functional domains: the reactive site for plasmin, the plasmin(ogen) or LBS-binding site (complementary to the LBS [lysine-binding site] of plasmin[ogen]), and the cross-linking site for fibrin. These functional properties of α_2 PI have been reviewed recently.⁵ In the present study we have developed monoclonal antibodies to α_2 PI, and the epitopes recognized by these antibodies have been characterized, particularly in reference to the functional domains.

MATERIALS AND METHODS

Purified protein. α_2 PI was purified from human plasma by a previously described method.¹ The concentration was determined spectrophotometrically using $E_{1\text{cm}}^{1\%} = 7.03$ at 280 nm.¹

Native Glu-plasminogen and modified Lys-plasminogen were purified from human plasma by affinity chromatography using lysine-sepharose followed by diethyl aminoethyl Sephadex ion-exchange chromatography as described.⁸ The concentration of plasminogen was determined spectrophotometrically using $E_{1\text{cm}}^{1\%} = 16.8$ at 280 nm.⁹

Human fibrinogen prepared according to the method of Blombäck

and Blombäck¹⁰ was further purified by removing contaminating plasminogen and plasma fibronectin from the preparation with lysine-Sepharose⁸ and gelatin-Sepharose¹¹ respectively. The concentration was determined spectrophotometrically using $E_{1\text{cm}}^{1\%} = 15.1$ at 280 nm, and the coagulatable protein was more than 95%.

Plasmin was prepared by the activation of plasminogen using urokinase-coupled Sepharose 4B as described.¹ Factor XIII was purified from human plasma by the method described by Curtis and Lorand.¹² Factor XIII activity was assayed by the dansylcadaverine incorporation method as described by Lorand et al.¹³ One unit of factor XIII is defined as the activity of factor XIII present in 1 mL of normal pooled standard plasma.

Purification of the α_2 PI-plasmin complex. α_2 PI-plasmin complex was prepared in an α_2 PI excess according to the method of Wiman and Collen.^{14,15} The α_2 PI-plasmin complex was isolated using lysine-Sepharose as described. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the complex showed protein bands at 150,000 daltons (150 K) and 11 K. The 11-K fragment is the C-terminal fragment of α_2 PI, which was originally observed by Wiman and Collen¹⁵ as an 8-K fragment that was cleaved from α_2 PI upon the α_2 PI-plasmin complex formation but which remained firmly bound to the complex. This fragment could be dissociated from the complex only in the presence of denaturants such as SDS.¹⁵ The α_2 PI-plasmin complex was also prepared by the same method using ¹²⁵I-labeled plasminogen, and the molar concentration of the complex was calculated from the radioactivity of the complex and the specific radioactivity of the original plasminogen used, assuming one-to-one molar complex formation. One $\mu\text{mol/L}$ of the complex was found to have an absorbance value of 0.157 at 280 nm.

Synthetic peptides. The synthetic C-terminal 26-residue peptide of α_2 PI¹⁶ (H-Gly-Asp-Lys-Leu-Phe-Gly-Pro-Asp-Leu-Lys-Leu-Val-Pro-Pro-Met-Glu-Glu-Asp-Tyr-Pro-Gln-Phe-Gly-Ser-Pro-Lys-OH) was a gift from the Research Institute, Daiichi Pharmaceutical Co (Tokyo). The synthetic N-terminal 12-residue peptide of α_2 PI¹⁷ (H-Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Leu-Lys-OH) was synthesized by solid-phase peptide synthesis using a peptide synthesizer, model 430 A (Applied Biosystems Inc, Foster City, CA).

Reagents for monoclonal antibody production. The mouse myeloma cell line P3U1 was provided by Dr T. Watanabe, Kyushu University, Japan. The following reagents were purchased: Freund's adjuvants, polyethylene glycol 1540, and 2, 6, 10, 14-tetramethyl pentadecane (pristane) from Wako Chemical Co (Osaka, Japan); Dulbecco's modified Eagle's medium from GIBCO (Chagrin Falls, OH); fetal calf serum from Filtron (Victoria, Australia); and

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hypoxanthine, aminopterin, thymidine (HAT), gelatine, diisopropylfluorophosphate and bovine serum albumin (BSA) from Sigma Chemical Co (St Louis). Other reagents were from Sigma or Wako, and were analytic grade.

Monoclonal antibody production. Monoclonal antibodies against α_2 PI were produced according to the method of Köhler and Milstein.¹⁸ Four-week-old female BALB/C mice were immunized with human α_2 PI three times. First, 50 μ g in complete Freund's adjuvant was injected intraperitoneally. Second, 50 μ g in incomplete Freund's adjuvant was injected intraperitoneally. Third, 30 μ g in phosphate-buffered saline was injected intravenously. Spleen cells from immunized mice were fused with the mouse myeloma cell lines P3-x63-Ag8-U1 (P3U1) by using 50% polyethylene glycol 1540. The fused cells were cultured in HAT media. Anti- α_2 PI antibody-producing cell lines, screened by an enzyme-linked immunosorbent assay (ELISA), were recloned by limiting dilution. Finally, each clone was injected intraperitoneally into BALB/C mice that were pretreated with pristane. Monoclonal antibodies were purified from the ascites fluid of the mice by affinity chromatography using protein A-sepharose CL 4B (Pharmacia Fine Chemicals, Tokyo) according to the standard method.¹⁹

ELISA. ELISA was performed essentially according to previously described methods²⁰ and was used to screen antibody production of fused cells. α_2 PI (5 μ g/mL in 0.02 mol/L sodium phosphate and 0.135 mol/L NaCl, pH 7.4; phosphate-buffered saline)-coated polyvinylchloride microtiter plates (Titertek, Zwanenburg, The Netherlands) were first incubated with antibody sources at 37 °C for two hours and then with alkaline phosphatase-conjugated antimouse IgG rabbit IgG (1 μ g/mL) (Kikegaard & Perry Laboratory, Gaithersburg, MD) at 37 °C for two hours. The hydrolysis of p-nitrophenyl phosphate (1 mg/mL in 97 mmol/L diethanolamine, 0.49 mmol/L MgCl₂, pH 9.8) by microtiter plate-bound alkaline phosphatase was monitored at 25 °C using the ETY96 ELISA analyzer (Toko-Sokki, Tokyo). After each step the plates were washed with phosphate-buffered saline containing 1% BSA and 0.05% Tween 20 (washing buffer) five times. ELISA was also used to assay α_2 PI or the α_2 PI-plasmin complex in a purified system as follows. Monoclonal antibody (solid-phase antibody, 10 μ g/mL, in 50 mmol/L carbonate buffer, pH 9.6)-coated microtiter plates were incubated with various concentrations of α_2 PI or the α_2 PI-plasmin complex in washing buffer at 37 °C for four hours followed by alkaline phosphatase-conjugated monoclonal antibody (liquid-phase antibody) at 10 nmol/L in washing buffer at 37 °C for four hours. The enzymatic activity of each well was monitored as was described. The α_2 PI-plasmin complex in plasma was assayed using a modification of the method of Harpel.²¹ Immunopurified polyclonal rabbit antibody to human plasminogen was used as a solid-phase antibody (20 μ g/mL) and the alkaline phosphatase-conjugated monoclonal antibody JTPI-2 as a liquid-phase antibody (10 nmol/L). A standard curve was constructed using purified α_2 PI-plasmin complex.

Radioiodination of protein. α_2 PI, plasminogen, and monoclonal antibodies were radioiodinated using immobilized lactoperoxidase glucose oxidase (Enzymobead, Bio-Rad Laboratories, Richmond, CA) and Na ¹²⁵I (17 Ci/mg) (New England Nuclear, Boston). The concentration of radiolabeled protein was determined spectrophotometrically. The antiplasmin activity of radiolabeled α_2 PI was measured as will be described in the section entitled "Effect of monoclonal antibodies on antiplasmin activity of α_2 PI" and was the same as that of native α_2 PI.

Binding study of monoclonal antibodies. The avidities of ¹²⁵I-labeled antibodies for α_2 PI and α_2 PI-plasmin complexes were assessed by a solid-phase assay previously described by Frankel and Gerhard.²² α_2 PI or the α_2 PI-plasmin complex was dissolved to a concentration of 100 nmol/L in phosphate-buffered saline. Each

well of the polyvinylchloride microtiter plate was coated with 100 μ L of α_2 PI or the α_2 PI-plasmin complex and was incubated at 4 °C for 16 hours. The plate was washed five times with washing buffer. Various concentrations of ¹²⁵I-labeled antibodies were dissolved in washing buffer, 100 μ L of each antibody solution was added to each well, and the plate was allowed to incubate at 37 °C for four hours. After the plate was again washed with washing buffer five times, the wells were removed from the plate, and each well was subjected to γ counting using a γ counter (Packard Institute, Downers Grove, IL). The radioactivities of BSA-coated wells incubated with the same concentrations of ¹²⁵I-labeled antibodies were subtracted from the radioactivities of α_2 PI- or α_2 PI-plasmin complex-coated wells. The results for duplicate wells were averaged. The radioactivity found in each well was converted to the concentration of antibody bound. The concentration of free antibody was calculated from the total radioactivity of the antibody solution added to the well and the radioactivity of the antibody bound to the well. The dissociation constants of antibodies to α_2 PI or the α_2 PI-plasmin complex were calculated from a modified Scatchard plot according to the method of Frankel and Gerhard.²² The Sipsian heterogeneity constant for each monoclonal antibody to α_2 PI was also calculated as described.²²

Agarose isoelectric focusing. Isoelectric focusing of monoclonal antibodies was performed using Multiphor (LKB, Bromma, Sweden) at 4 °C over a pH range of 3.5 to 9.0 with 2.5% ampholine (LKB) according to the method described in the instructions of the manufacturer. After electrophoresis, the gel was fixed, dried, and stained with Coomassie blue.

SDS-PAGE. SDS-PAGE on a slab gel was carried out using a gradient separating gel of acrylamide (7.5% to 15%) containing 0.1% SDS and a stacking gel of 4% acrylamide containing 0.1% SDS according to the method of Laemmli.²³ Molecular weight standards of myosin (200 K), phosphorylase b (94 K), BSA (67 K), ovalbumin (45 K), carbonic anhydrase (30 K), trypsin inhibitor (22 K), cytochrome c (12.5 K), and aprotinin (6.5 K) were obtained from Bio-Rad Laboratories and Boehringer Mannheim GmbH (Mannheim, West Germany).

Immunoblotting technique. After separation by SDS-PAGE, the proteins were electrophoretically (50 V for 1.5 hours) transferred to nitrocellulose membranes (Bio-Rad) essentially as described²⁴ using an electroblot apparatus (Marysol Industry Co, Tokyo) equipped with a cooling system (Multitemp, LKB). After transferring, the nitrocellulose sheets were incubated in Tris buffer (0.02 mol/L Tris, 0.5 mol/L NaCl, pH 7.5) containing 3% (wt/vol) gelatin at 25 °C for two hours, washed twice with Tris buffer containing 0.05% Tween 20, and incubated in Tris buffer containing 1% (wt/vol) gelatin, 0.05% Tween 20, and monoclonal antibodies (500 ng/mL) at 25 °C for 16 hours. The nitrocellulose sheets were then washed with Tris buffer containing 0.05% Tween 20, incubated with horseradish peroxidase-conjugated antimouse IgG goat antibody (Bio-Rad) dissolved in Tris buffer containing 1% gelatin and 0.05% Tween 20 at 25 °C for two hours, washed twice with Tris buffer containing 0.05% Tween 20, and subjected to color development using 4-chloro-1-naphthol (Bio-Rad).

Radioautography. After SDS-PAGE of the samples containing radioiodinated proteins, the slab gel was dried and then exposed to x-ray film (Lo-dose film, E.I. Du Pont de Nemours & Co, Wilmington, DE) in the presence of intensifying screens (Lo-Dose/2 mammography intensifying screen, Du Pont) for 48 hours at -70 °C.

Effect of monoclonal antibodies on antiplasmin activity of α_2 PI. α_2 PI was mixed and incubated with various concentrations of monoclonal antibodies or buffered saline at 37 °C for 30 minutes and then at 4 °C for 24 hours. Subsequently, 100 μ L of the α_2 PI (12.5 nmol/L) and 100 μ L of plasmin (25 nmol/L) were mixed in 500 μ L of Tris-buffered saline (0.05 mol/L Tris, 0.1 mol/L NaCl, pH 7.4). After incubation for zero, 30 seconds, 2 minutes, 5 minutes,

and 30 minutes, 500 μ L of S-2251 (plasmin-specific tripeptide substrate H-D-Val-Leu-Lys-*p*-nitroanilide, Daiichi Pure Chemicals, Tokyo) (2 mmol/L) was added, and the subsequent hydrolysis of S-2251 by residual plasmin was monitored using a DU-8 spectrophotometer (Beckman Instruments, Fullerton, CA).^{25,26} The antiplasmin activity of α_2 PI preincubated with monoclonal antibody was expressed as a percentage of that of α_2 PI without monoclonal antibody using the standard curve constructed from the activities of various concentrations of α_2 PI.

Effect of monoclonal antibodies on cross-linking of α_2 PI to fibrin. ¹²⁵I-labeled α_2 PI was incubated with various concentrations of monoclonal antibodies or buffered saline at 37 °C for 30 minutes and then at 4 °C for 24 hours. Subsequently, 10 μ L of the ¹²⁵I-labeled α_2 PI (700 μ g/mL), 10 μ L of fibrinogen (20 mg/mL), 10 μ L of factor XIII (5 U/mL), 20 μ L of thrombin (5 U/mL), and 50 μ L of Tris-buffered saline containing 5 mmol/L CaCl₂, 2% BSA, and 20 U/mL aprotinin (Mochida Pharmaceuticals, Tokyo) were mixed and incubated at 37 °C. Ten or 30 minutes later, the clots were squeezed with a stick and washed three times with Tris-buffered saline containing 2% BSA, 5 mmol/L EDTA, and aprotinin (10 U/mL).²⁷ The washed clots were then redissolved by heating at 100 °C in 8 mol/L urea, 2% SDS, and 10% β -mercaptoethanol. From the radioactivity of the redissolved clot and the total radioactivity of each initial reaction mixture, the percentage of α_2 PI cross-linked to fibrin was calculated.

Effect of monoclonal antibodies on the inhibition of plasminogen adsorption to fibrin by α_2 PI. α_2 PI was incubated with various concentrations of monoclonal antibodies or buffered saline at 37 °C for 30 minutes and then at 4 °C for 24 hours. Subsequently, 10 μ L of the α_2 PI (700 μ g/mL), 10 μ L of ¹²⁵I-labeled Lys-plasminogen (2 mg/mL), 10 μ L of fibrinogen (20 mg/mL), 20 μ L of thrombin (5 U/mL), and Tris-buffered saline containing 2% BSA, 2 mmol/L EDTA, and 20 U/mL aprotinin were mixed and incubated at 37 °C for two hours. Clots were squeezed using a stick and washed three times with Tris-buffered saline containing 2% BSA, 2 mmol/L EDTA, and aprotinin (10 U/mL). The clots were redissolved by heating at 100 °C in 8 mol/L urea, 2% SDS, and 10% β -mercaptoethanol. The adsorption of ¹²⁵I-labeled plasminogen to fibrin was calculated from the initial radioactivity and the radioactivity of the redissolved clot.²⁸ The degrees of adsorption of ¹²⁵I-labeled plasminogen at various concentrations of α_2 PI preincubated with monoclonal antibodies were compared with those without monoclonal antibodies.

Effect of synthetic peptides on the binding of α_2 PI to monoclonal antibodies. Polyvinylchloride microtiter plates were coated with monoclonal antibody at 10 μ g/mL in 0.05 mol/L carbonate buffer, pH 9.6, at 4 °C and were further incubated with 0.05 mol/L carbonate buffer, pH 9.6, containing 1% BSA for two hours at 37 °C. The plates were washed with washing buffer five times. ¹²⁵I-labeled α_2 PI at the concentration of the half-saturated binding level for each antibody was mixed with various concentrations of the C-terminal or N-terminal peptide and was allowed to incubate in antibody-coated wells at 37 °C for four hours. After washing, the wells were removed from the plates and were subjected to γ counting. The binding of ¹²⁵I- α_2 PI with various concentrations of the peptides was expressed as the percentage of that without the peptides.

RESULTS

Hybridomas producing monoclonal antibodies against α_2 PI were isolated. After limiting dilution, 26 clones were shown to produce antibodies capable of binding to α_2 PI. Three clones were selected for further investigation and were named JTPI-1, JTPI-2, and JTPI-3.

The purified monoclonal antibodies from JTPI-1, JTPI-2, and JTPI-3 each showed a single band (isoelectric point) upon isoelectric focusing (Fig 1). The class and subclass of each monoclonal antibody were determined by an immunodiffusion test using rabbit antibodies specific for murine immunoglobulins. The subclass of heavy chain and class of light chain for JTPI-1, JTPI-2, and JTPI-3 were γ 1 $\cdot$ κ , γ 2a $\cdot$ κ , and γ 2b $\cdot$ κ , respectively.

The binding sites of the monoclonal antibodies to α_2 PI were investigated by immunoblotting (Fig 2). JTPI-1 bound α_2 PI (67 K) but was not shown to bind the α_2 PI-plasmin complex (150 K) or the α_2 PI fragment (11 K) that was cleaved from α_2 PI upon complex formation. JTPI-2 bound α_2 PI (67 K) and the α_2 PI fragment (11 K). It did not bind the α_2 PI-plasmin complex (150 K). JTPI-3 bound α_2 PI (67 K) and the α_2 PI-plasmin complex (150 K). It did not bind the α_2 PI fragment (11 K). When the α_2 PI-plasmin complex was incubated with a catalytic amount of plasmin, the complex (150 K) gradually lost its ability to bind JTPI-3 (Fig 3B), although any change in its molecular weight was not apparent (Fig 3A).

The effect of monoclonal antibodies on the antiplasmin activity of α_2 PI was studied. The presence of a molar excess of JTPI-1 totally inhibited the antiplasmin activity of α_2 PI, whereas JTPI-2 and JTPI-3 did not inhibit the antiplasmin activity of α_2 PI at all (Fig 4). A half-maximum inhibition of the antiplasmin activity of α_2 PI by JTPI-1 was obtained at the molar ratio (antibody: α_2 PI) of 0.3 (Fig 5), whereas neither JTPI-2 nor JTPI-3 showed any inhibition of the antiplasmin activity of α_2 PI even at a molar ratio of 100 (Fig 5).

During the inhibition of plasmin by α_2 PI, α_2 PI reacts with plasmin to form an enzymatically inactive complex. This

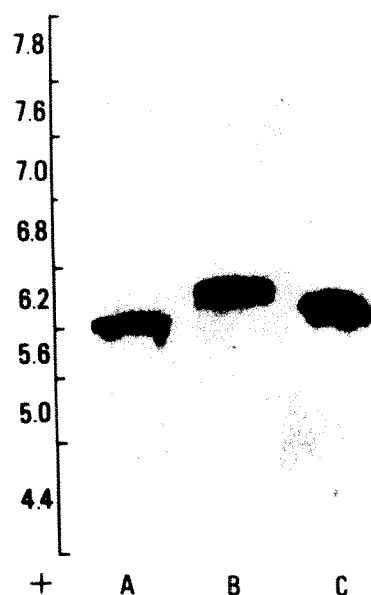


Fig 1. Isoelectric focusing of purified monoclonal antibodies. Twenty micrograms of each monoclonal antibody was subjected to agarose isoelectric focusing at 4 °C on a 3.5- to 9.0-pH gradient as described in Materials and Methods. A, B and C correspond to the antibodies JTP-1, -2, and -3 respectively.

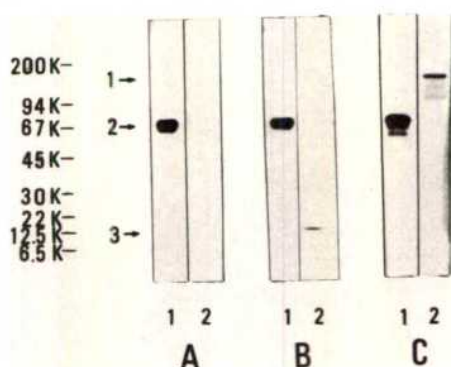


Fig 2. Immunoblotting of α_2 PI and the α_2 PI-plasmin complex using monoclonal antibodies to α_2 PI. Two hundred nanograms of α_2 PI (lane 1) or 400 ng of the α_2 PI-plasmin complex (lane 2) were electrophoresed without reduction by SDS-PAGE on a slab gel with gradient (7.5% to 15%) acrylamide and 0.1% SDS, and the proteins were transferred to nitrocellulose membrane. Strips of the membrane were incubated with the respective monoclonal antibodies JTPI-1 (A), JTPI-2 (B), and JTPI-3 (C). The strips were then washed and incubated with peroxidase-conjugated anti-mouse IgG goat antibody, and color was developed with 4-chloro-1-naphthol as described in Materials and Methods. Arrow 1, α_2 PI-plasmin complex (150 K); arrow 2 α_2 PI (67 K); arrow 3, α_2 PI fragment (11 K). On the left is a molecular weight calibration scale constructed using molecular weight standards.

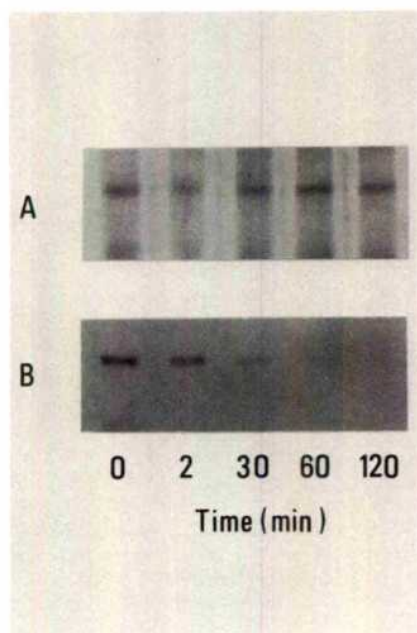


Fig 3. Immunoblotting of the α_2 PI-plasmin complex undergoing plasminic degradation. A quantity of 1 μ mol/L of the α_2 PI-plasmin complex was incubated with 20 nmol/L plasmin in Tris-buffered saline at 37 °C. After incubation for 2, 30, 60, and 120 min, 10- μ L aliquots were removed. The samples were subjected to SDS-PAGE (A) followed by immunoblotting using antibody JTPI-3 (B) as described in Fig 2.

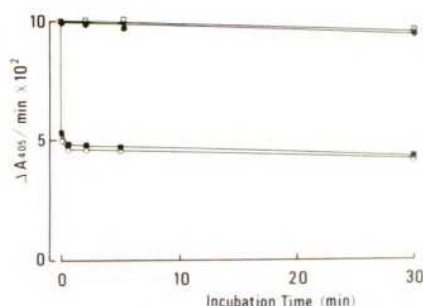


Fig 4. Effects of monoclonal antibodies on the antiplasmin activity of α_2 PI. One hundred microliters of α_2 PI (12.5 nmol/L) preincubated with monoclonal antibodies (125 nmol/L) and 100 μ L of plasmin (25 nmol/L) were mixed in 500 μ L of Tris-buffered saline and incubated at 25 °C. After various incubation periods, the residual plasmin activity was assayed as described in Materials and Methods. The amidolysis activities of plasmin without α_2 PI ($\square$), plasmin with α_2 PI ($\circ$), plasmin with α_2 PI preincubated with JTPI-1 ($\bullet$), and plasmin with α_2 PI preincubated with JTPI-2 or JTPI-3 ($\blacksquare$) were plotted.

complex formation was observed by radioautography after SDS-PAGE using radioactive α_2 PI. This ^{125} I- α_2 PI-plasmin complex (Fig 6, lane 2) gave rise to radioactive zones in the gel corresponding to the complex (150 K), a smear between the complex (150 K) and the modified α_2 PI (55 K), and modified α_2 PI (55 K). These zones were caused by dissociation of α_2 PI from the complex during electrophoresis. Complex formation was not seen when α_2 PI was preincubated with a molar excess of JTPI-1 (Fig 6). When complex formation was inhibited by JTPI-1 α_2 PI (67 K) was degraded to a 60-K molecule by the free unreacted plasmin (Fig 6). The binding of this degraded α_2 PI to monoclonal antibodies was examined by immunoblotting. The immunoblotting showed binding of the degraded α_2 PI to JTPI-3 but did not show any binding to JTPI-2 (Fig 7).

The influence of monoclonal antibodies on the cross-linking of α_2 PI to fibrin and on the inhibitory activity of α_2 PI for plasminogen adsorption to fibrin was assessed. JTPI-1, JTPI-2, and JTPI-3 had no influence on these functions of α_2 PI even when the antibodies were used in a tenfold molar

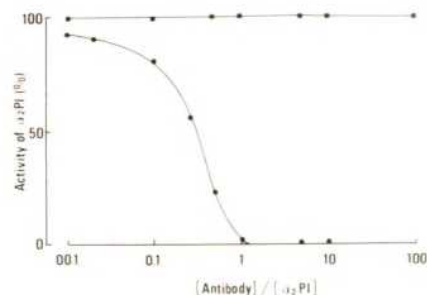


Fig 5. Effects of various concentrations of monoclonal antibodies on the antiplasmin activity of α_2 PI. The antiplasmin activity of α_2 PI preincubated with various concentrations of monoclonal antibody JTPI-1 ($\bullet$), JTPI-2, or -3 ($\blacksquare$) was assessed at zero-time incubation with plasmin as in Fig 4 and expressed as a percentage of that of α_2 PI without antibody. The activities of α_2 PI were plotted against the molar ratios of monoclonal antibodies to α_2 PI plotted on a semilogarithmic scale.

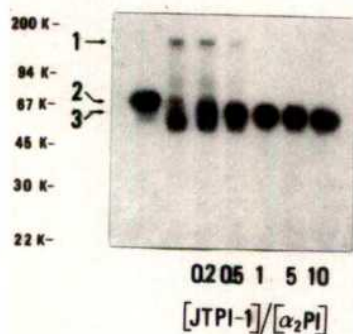


Fig 6. Radioautogram of the reaction products of plasmin and ^{125}I - $\alpha_2\text{PI}$ preincubated with monoclonal antibody JTPI-1. Twenty-five microliters of ^{125}I - $\alpha_2\text{PI}$ (2 $\mu\text{mol/L}$) preincubated with various concentrations of the antibody in Tris-buffered saline was mixed with 25 μL of plasmin (2 $\mu\text{mol/L}$). After five minutes' incubation at 37 $^\circ\text{C}$, the samples were mixed with 0.125 mol/L Tris, pH 6.8, containing 2% SDS and heated at 100 $^\circ\text{C}$ for two minutes. The samples were subjected to SDS-PAGE and analyzed by radioautography as described in Materials and Methods. The first column (extreme left), native $\alpha_2\text{PI}$; the second column, $\alpha_2\text{PI}$ reacted with plasmin. From the third column to the right, $\alpha_2\text{PI}$ preincubated with the antibody JTPI-1 in various molar ratios of JTPI-1/ $\alpha_2\text{PI}$ as indicated was reacted with plasmin. Arrow 1, $\alpha_2\text{PI}$ -plasmin complex; arrow 2, native $\alpha_2\text{PI}$; arrow 3, degraded $\alpha_2\text{PI}$. On the left is a molecular weight calibration scale constructed using molecular weight standards.

excess over $\alpha_2\text{PI}$. Furthermore, a synthetic C-terminal peptide of $\alpha_2\text{PI}$ (26 residues), which contains the plasmin(ogen) or LBS-binding site of $\alpha_2\text{PI}$, did not inhibit the binding of ^{125}I -labeled $\alpha_2\text{PI}$ to each monoclonal antibody-coated microtiter plate (Fig 8). However, the N-terminal peptide (12 residues), which contains the cross-linking site, inhibited the binding of ^{125}I -labeled $\alpha_2\text{PI}$ to JTPI-3-coated microtiter plates (Fig 8).

Dissociation constants (KD) for monoclonal antibodies to $\alpha_2\text{PI}$ or the $\alpha_2\text{PI}$ -plasmin complex were assessed to estimate the avidity of these monoclonal antibodies (summarized in Table 1). The dissociation constants for $\alpha_2\text{PI}$ binding to all three monoclonals were the same, 1.54 to 2.89 nmol/L. The KDs for JTPI-2 and JTPI-3 binding to the $\alpha_2\text{PI}$ -plasmin complex were also 2.59 to 3.17 nmol/L. However, the KD for JTPI-1 binding to the $\alpha_2\text{PI}$ -plasmin complex was markedly higher, 10.6 nmol/L. Sipsian heterogeneity constants²² for JTPI-1, JTPI-2, and JTPI-3 were essentially equal and were 1.10, 0.99, and 0.99, respectively.

ELISA of $\alpha_2\text{PI}$ and the $\alpha_2\text{PI}$ -plasmin complex was performed using JTPI-1 as a solid-phase antibody and alkaline phosphatase-conjugated JTPI-2 as a liquid-phase antibody. As shown in Fig 9, the enzymatic activities of plates increased proportionally to the $\alpha_2\text{PI}$ concentration from 0.8 to 3 nmol/L. But when the $\alpha_2\text{PI}$ -plasmin complex was assayed in the same way, no enzymatic activities were

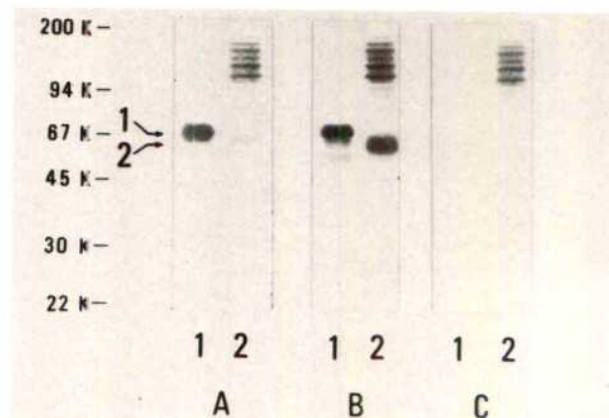


Fig 7. Immunoblotting of the reaction products of plasmin and $\alpha_2\text{PI}$ preincubated with monoclonal antibody JTPI-1. Lane 1 contains control native $\alpha_2\text{PI}$ only. In lane 2, $\alpha_2\text{PI}$ preincubated with JTPI-1 at the molar ratio (JTPI-1/ $\alpha_2\text{PI}$) of 1 was reacted with plasmin and subjected to SDS-PAGE as in Fig 6. Proteins transferred to nitrocellulose sheets were reacted with JTPI-2 (A), JTPI-3 (B), or Tris-buffered saline (C) followed by incubation with enzyme-conjugated antimouse IgG goat antibody and color development as described in Materials and Methods. Arrow 1, native $\alpha_2\text{PI}$; arrow 2, degraded $\alpha_2\text{PI}$. The multiple bands seen in upper portions of lane 2 were plasmin-digested monoclonal antibody JTPI-1 that was used for preincubation of $\alpha_2\text{PI}$ and reacted with enzyme-conjugated antimouse IgG antibody. On the left is a molecular weight calibration scale constructed using molecular weight standards.

detected up to 5 nmol/L. The influence of the $\alpha_2\text{PI}$ -plasmin complex on ELISA of $\alpha_2\text{PI}$ was investigated. When a mixture of a fixed concentration of $\alpha_2\text{PI}$ (2 nmol/L) and various concentrations of the $\alpha_2\text{PI}$ -plasmin complex was assayed by ELISA in the same way, no significant increase in enzymatic activity caused by the presence of the complex was detected until the concentration of the complex was increased up to more than 5 nmol/L (Fig 10).

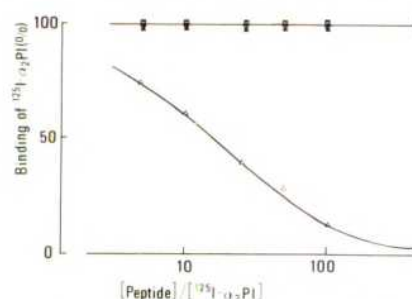


Fig 8. Effects of the synthetic peptides on the binding of monoclonal antibodies to $\alpha_2\text{PI}$. ^{125}I - $\alpha_2\text{PI}$ at the half-saturated level with various concentrations of synthetic C-terminal or N-terminal peptide was added to antibody-coated wells, and the plates were allowed to incubate at 37 $^\circ\text{C}$ for four hours. After washing, the wells were removed from the plates and were subjected to γ counting. Binding of ^{125}I - $\alpha_2\text{PI}$ to antibody-coated wells in the presence of the C-terminal peptide (JTPI-1 [●], JTPI-2 [■], JTPI-3 [▲]) or to antibody-coated wells in the presence of the N-terminal peptide (JTPI-1 [○], JTPI-2 [□], JTPI-3 [△]) was expressed as a percentage of that in the absence of the peptide and plotted against the molar ratios of the peptide to $\alpha_2\text{PI}$ plotted on a semilogarithmic scale.

Table 1. KD and Heterogeneity Constant (α)

	α_2 PI		α_2 PI-Plasmin
	KD (mol/L)	α	KD (mol/L)
JTPI-1	2.18×10^{-9}	1.10	1.06×10^{-8}
JTPI-2	2.89×10^{-9}	0.99	3.17×10^{-9}
JTPI-3	1.54×10^{-9}	0.99	2.59×10^{-9}

The KD values for monoclonal antibodies to α_2 PI or the α_2 PI-plasmin complex and Sipsian heterogeneity constants were calculated as described in Materials and Methods.

Concentrations of α_2 PI and the α_2 PI-plasmin complex in urokinase-treated plasma were assayed by ELISA using various antibodies for the solid-phase antibody (Fig 11). The concentration of the α_2 PI-plasmin complex (measured by ELISA using antiplasminogen for the solid-phase antibody) increased upon the addition of urokinase to the plasma, whereas the concentration of free α_2 PI (measured by ELISA using JTPI-1 for the solid-phase antibody) decreased. The total amount of α_2 PI (free α_2 PI plus the α_2 PI-plasmin complex) remained constant ($0.93 \pm 0.02 \mu\text{mol/L}$). When the experiment was repeated using JTPI-3 for the ELISA solid-phase antibody, the amount of α_2 PI detected (JTPI-3 detects free α_2 PI and the α_2 PI-plasmin complex) remained unchanged upon urokinase treatment.

DISCUSSION

The plasmin(ogen) molecule has specific sites where it noncovalently binds to α_2 PI or fibrin. The sites are called LBS. Their complementary site in α_2 PI could be called the LBS binding site and is located in the region close to the C-terminal end.¹⁶ When α_2 PI inhibits plasmin, the initial reaction involves the binding of the LBS of plasmin to the LBS binding site of α_2 PI. This reaction is reversible. Then, a firm covalent bond is formed between the active-site serine residue of plasmin and the reactive-site leucine residue of α_2 PI¹⁵ to generate the α_2 PI-plasmin complex. Upon complex formation, a C-terminal fragment, which is shown in the present study to be 11 K, is cleaved from α_2 PI but remains noncovalently bound to the complex.¹⁵ The LBS binding site of α_2 PI not only acts during inhibition of plasmin but also inhibits adsorption of plasminogen to fibrin by competing with fibrin for plasminogen binding. Inhibition of plasminogen adsorption to fibrin retards the initiation of the fibrino-

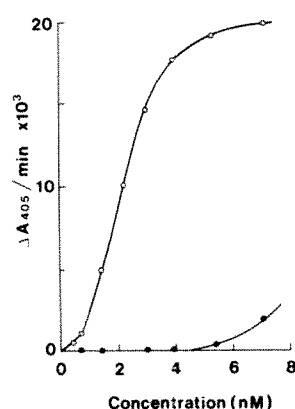


Fig 9. ELISA of α_2 PI and the α_2 PI-plasmin complex. α_2 PI (○) or the α_2 PI-plasmin complex (●) was assayed by ELISA using JTPI-1 for the solid-phase antibody and alkaline phosphatase-conjugated JTPI-2 for the liquid-phase antibody as described in Materials and Methods. The concentrations of α_2 PI or the complex are on the abscissa, and the enzyme activity is on the ordinate.

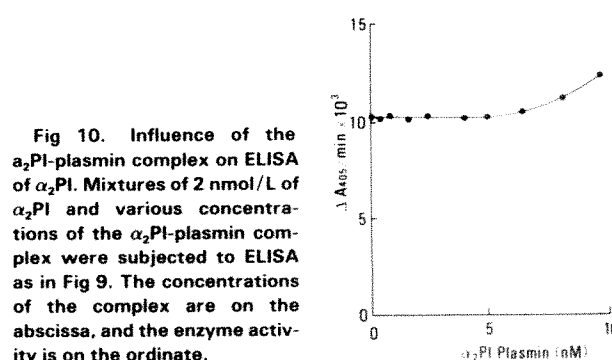


Fig 10. Influence of the α_2 PI-plasmin complex on ELISA of α_2 PI. Mixtures of 2 nmol/L of α_2 PI and various concentrations of the α_2 PI-plasmin complex were subjected to ELISA as in Fig 9. The concentrations of the complex are on the abscissa, and the enzyme activity is on the ordinate.

lytic process.⁶ On the other hand, α_2 PI itself binds to fibrin during blood coagulation when activated coagulation factor XIII (transglutaminase) catalyzes a cross-linking reaction between α_2 PI and the α chain of fibrin.^{27,29} Studies have revealed that fibrin is mainly protected from plasmin attack by α_2 PI cross-linked to the α chain of fibrin³⁰ during the physiologically occurring fibrinolytic process. Thus, α_2 PI has three functional domains, the reactive site, the LBS binding site, and the cross-linking site. The reactive site is in the C-terminal region of α_2 PI.¹⁵ The LBS binding site is close to the C-terminal end and is contained in the 11-K C-terminal fragment.¹⁶ The cross-linking site is the glutamine located in the second position from the N-terminal end.²⁹ In the present study, we have developed monoclonal antibodies to α_2 PI and have studied the epitopes recognized by these antibodies in relation to the functional domains of α_2 PI.

From 26 clones that produced anti- α_2 PI antibodies, three clones were selected and were named JTPI-1, JTPI-2, and JTPI-3. Each antibody showed a single isoelectric point (Fig 1) and consisted of one subclass of heavy chain and one class

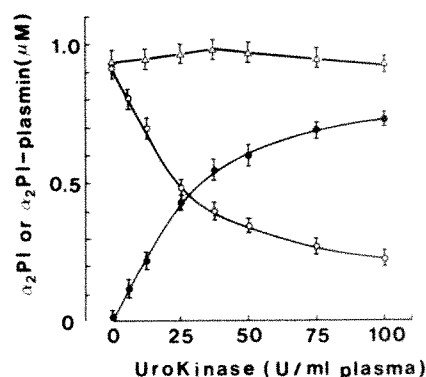


Fig 11. Assay of α_2 PI and the α_2 PI-plasmin complex in plasma. Various concentrations of urokinase were added to citrated plasma in vitro. After a one-hour incubation at 37 °C, aprotinin (115 U/mL) and diisopropyl fluorophosphate (1 mmol/L) were added to the plasma. Then the plasma was diluted 400-fold in washing buffer and assayed for α_2 PI and the α_2 PI-plasmin complex by ELISA as described in Materials and Methods. α_2 PI assayed using JTPI-1 as a solid-phase antibody, ○; α_2 PI assayed using JTPI-3 as a solid-phase antibody, △; α_2 PI-plasmin complex assayed using polyclonal antiplasminogen antibody as a solid-phase antibody, ●. Alkaline phosphatase-conjugated JTPI-2 was used as a liquid-phase antibody. Each point represents the mean $\pm$ SE of triplicate determinations.

of light chain. These data in addition to the Sipsian heterogeneity constants being approximately equal to 1 (Table 1) demonstrate the monoclonality of each antibody.

When the binding site of JTPI-1 was analyzed by immunoblotting, JTPI-1 bound native α_2 PI (67 K) but did not show binding to either the α_2 PI-plasmin complex (150 K) or the C-terminal fragment (11 K). When the binding of radiolabeled antibodies to solid-phase α_2 PI or α_2 PI-plasmin complex was analyzed, JTPI-1 was shown to bind the α_2 PI-plasmin complex but with a markedly lower avidity than that for α_2 PI alone; KD for the complex was five times higher than that for α_2 PI alone (Table 1). Since JTPI-1 completely inhibits the antiplasmin activity of α_2 PI (Figs 4 and 5) by interfering with the formation of α_2 PI-plasmin complexes (Fig 6), the epitope recognized by this antibody must be located either in the reactive site or in the region very close to the reactive site. Although JTPI-1 inhibits complex formation by blocking the reactive site of α_2 PI, the epitope for JTPI-1 on preformed α_2 PI-plasmin complexes remains available but has a markedly reduced affinity (increased KD value) for the antibody (Table 1). The increase in the KD value of JTPI-1 caused by α_2 PI-plasmin complex formation suggests that conformational changes occurred in the epitope upon complex formation or that the plasmin molecule bound to α_2 PI became a steric hindrance, thus preventing access of the antibody to the epitope.

When the ^{125} I- α_2 PI plasmin complex was analyzed by SDS-PAGE (Fig 6), it gave rise to radioactive zones in the gel corresponding to the complex (150 K), a smear (55 to 150 K), and the modified α_2 PI (55 K). These zones were thought to be caused by dissociation of the complex during SDS-PAGE performed by the procedure of Laemmli.²³ The similar dissociation of another complex (plasminogen activator-plasminogen activator-inhibitor complex) during SDS-PAGE performed by Laemmli's procedure has been reported.³¹

When α_2 PI-plasmin complex formation was inhibited by JTPI-1, plasmin that remained free rapidly degraded α_2 PI (Fig 6). The estimated molecular weight of the degraded α_2 PI was approximately 7 K less than that of α_2 PI, and this degraded form of α_2 PI bound to JTPI-3 but not to JTPI-2 upon immunoblotting (Fig 7). Since JTPI-2 recognizes an epitope in the C-terminal 11-K fragment (see the following paragraph), these findings suggest that free α_2 PI is very susceptible to plasmin degradation and that a susceptible bond is located in the C-terminal region of the molecule.

JTPI-2 bound α_2 PI and the α_2 PI-plasmin complex equally well (Table 1). Immunoblotting data showed that JTPI-2 bound to the C-terminal 11-K fragment (Fig 2). The fact that JTPI-2 recognizes the C-terminal 11-K fragment in α_2 PI-plasmin complexes as well as α_2 PI confirms the previous finding that the C-terminal fragment cleaved from α_2 PI upon α_2 PI-plasmin complex formation remains firmly bound to the complex and can be separated only in the presence of denaturants such as SDS.¹⁵ Although the C-terminal 11-K fragment contains the LBS binding site, the epitope recognized by JTPI-2 is separate from the LBS binding site for the following reasons: JTPI-2 did not inhibit either the binding of plasminogen to fibrin or the immediate-type antiplasmin

activity of α_2 PI at all (Figs 4 and 5), and the C-terminal 26-residue peptide that contains the LBS binding site (plasminogen-binding site) failed to inhibit binding of the antibody to α_2 PI (Fig 8). Thus the epitope for JTPI-2 seems to be located between the reactive site and the LBS binding site.

JTPI-3 bound to α_2 PI-plasmin complexes (150 K) and had no effect on the following functions of α_2 PI: antiplasmin activity (Figs 4 and 5), interference with plasminogen binding to fibrin, and cross-linking to fibrin. Although JTPI-3 did not inhibit the cross-linking of α_2 PI to fibrin, the binding of JTPI-3 to α_2 PI was inhibited by the N-terminal 12-residue peptide (Fig 8) that contains the cross-linking site.²⁹ These results suggest that the epitope for JTPI-3 is located somewhere between the cross-linking site, the glutamine in the second position from the N-terminal end, and the 12th-position lysine. When the α_2 PI-plasmin complex was incubated with plasmin, the complex gradually lost its capacity to bind JTPI-3 (Fig 3B), although any change in its molecular weight was not apparent (Fig 3A). These findings together with the suggestion that JTPI-3 recognizes the N-terminal region of α_2 PI may support the proposal that plasmin degrades the complex by cleavage of a peptide near the N-terminus of the α_2 PI component of the complex.^{15,23}

The markedly lower affinity of JTPI-1 to the α_2 PI-plasmin complex than to free α_2 PI (Table 1) provided the ability to measure free α_2 PI without significant influence from the α_2 PI-plasmin complex (Figs 9 and 10). In dilute plasma (1:400 dilution) the concentration of the α_2 PI-plasmin complex (<2.5 nmol/L) is below the range of detection for JTPI-1. Therefore an ELISA to the dilute plasma similar to that used in Fig 9 should detect only free α_2 PI.

When plasma is treated with urokinase, the plasmin that is generated immediately forms complexes with α_2 PI. At low concentrations of urokinase, α_2 PI-plasmin complex formation is proportional to the concentration of urokinase.²¹ Figure 11 demonstrates that the concentration of the α_2 PI-plasmin complex (detected by antiplasminogen) increased upon urokinase treatment, whereas the concentration of free α_2 PI (detected by JTPI-1) decreased. The total amount of α_2 PI (free α_2 PI plus the α_2 PI-plasmin complex) remained constant. Additionally, when the same plasma samples were assayed using JTPI-3 for detection (JTPI-3 detects both free α_2 PI and the α_2 PI-plasmin complex), the amount of α_2 PI detected was not influenced by urokinase treatment (Fig 11). These results indicate that under appropriate conditions the level of α_2 PI detected by JTPI-1 represents free α_2 PI.

Currently available immunologic assays for α_2 PI (such as single radial immunodiffusion, electroimmunoassay, and ELISA using polyclonal antibodies) measure not only α_2 PI but also the α_2 PI-plasmin complexes. In this respect, the present method for detection of free α_2 PI has a definite advantage because it allows an accurate determination of the α_2 PI concentration in plasma from patients such as those with disseminated intravascular coagulation or receiving thrombolytic therapy in whom the levels of the α_2 PI-plasmin complex in plasma are often markedly increased.^{5,32} The combination of an ELISA that measures free α_2 PI (detection by JTPI-1) and an ELISA that measures the α_2 PI-plasmin complex (detection by antiplasminogen) allows accurate

information to be determined about the behavior of α_2 PI in various disease states.

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Epidemiological Investigation of the Prevalence of von Willebrand's Disease

By Francesco Rodeghiero, Giancarlo Castaman, and Enrico Dini

To evaluate the prevalence of von Willebrand's disease (vWd) we carried out an epidemiological investigation among school children of the Veneto region in northern Italy. A total of 1,218 of 1,281 possible children participated in the study. They were 11 to 14 years of age, and all attended secondary schools in two distinct small areas, 70 km apart, between which there is no social contact. A blood sample was taken from each subject for determination of the blood group and von Willebrand factor (vWf) level (measured as ristocetin cofactor and expressed in IU/dL after calibration of the internal pool against an international standard), and the parents were given a questionnaire concerning hemorrhagic symptoms in the members of the family in the last three generations. Separate normal ranges were calculated for blood group O and non-O subjects (1,166 children and 289 adults) with a nonparametric method because the distribution curves of the reference values did not fit the gaussian distribution.

VON WILLEBRAND'S DISEASE (vWd) is an autosomally inherited disorder resulting from a quantitative or a qualitative defect of von Willebrand factor (vWf).¹ This protein circulates in plasma complexed to factor VIII (VIII/vWf complex) and is required for the normal adhesion of platelets to subendothelium² and for in vitro ristocetin-induced platelet aggregation.³ Clinical expressions of the disease are variable. In addition to severe cases requiring replacement therapy to stop or prevent hemorrhages, there are mildly affected patients. Furthermore, some subjects carrying the mutant gene have no hemorrhagic symptoms.^{4,5}

The mutant gene has multiple phenotypic effects consisting essentially of a prolonged bleeding time and reduced factor VIII/vWf complex-related activities (factor VIII coagulant activity, vWf antigen, ristocetin cofactor activity of vWf).⁶ The bleeding time and VIII/vWf-related activities can be easily measured by laboratory tests, but the diagnosis of this disorder may be difficult because the laboratory data may overlap with the normal range and fluctuate with time in a given patient.⁷ Recently, the identification by multimeric sizing techniques of qualitative variants of vWd has added to the complexity of the diagnosis.⁸⁻¹²

So far, the prevalence of vWd has been estimated only from the cases registered at specialized centers and indicates

Diagnoses of vWd were considered only for children who had low vWf levels and were members of a family with a convincing bleeding history (case of "probable vWd"). A final diagnosis was assigned if, in addition to these criteria, at least one other family member on the side with hemorrhagic history had a low vWf level. Of the 1,218 children examined, ten were classified as having vWd (0.82%). Taking into account the 90% confidence interval for the lower limit of the normal range, this figure could range from 7 (0.57%) to 14 (1.15%). All these subjects were mildly to moderately affected and presented features of heterozygous classic vWd (type I). Affected subjects were distributed evenly in the two areas examined. Our results suggest that the prevalence of vWd might be much higher than previously reported and that a different screening approach might be of use for patients with mild bleeding diathesis

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a prevalence similar to or even lower than that of hemophilia.¹³⁻¹⁶ This paper reports the results of an epidemiological investigation of 1,218 children attending secondary school in two distinct areas of North Italy.

MATERIALS AND METHODS

Subjects. All 1,281 children aged 11 to 14 attending the three grades of secondary school in two distinct areas of the Veneto region (Province of Vicenza) were asked to participate in the investigation. The first area consists of two small neighboring towns 30 km north of Vicenza (Santorso and Piovene Rocchette, 11,676 inhabitants), and the second consists of two small neighboring towns 40 km south of Vicenza (Foiana Maggiore and Noventa Vicentina, 11,638 inhabitants). The possibility of investigating all resident children, the adequate size of the sample, and the administrative facilities caused us to choose these particular areas. Moreover, less than 10% of the two populations had a common ancestor, as judged by an analysis of family names, so that the two areas can be considered to be independent samples. Parents and physicians in charge of the children were given detailed information about the purpose and methodology of the investigation. All children for whom parents had provided written consent were admitted to the study.

Hemorrhagic symptoms were investigated through a questionnaire consisting of four sections that concerned the child under investigation, or her siblings, the father and his sibship and parents, and the mother and her sibship and parents. Specific symptoms investigated included epistaxis, hemorrhage after tooth extraction, hemorrhage after surgery, prolonged bleeding after superficial cuts, menorrhagia, postpartum hemorrhage, and easy bruising. Families for which a hemorrhagic history could be suspected were interviewed to get more detailed descriptions of reported bleeding episodes and additional information about relatives not specifically investigated in the questionnaire. Each symptom was recorded only when sufficiently fully reported and of not trivial importance. Easy bruising was considered only for male adults and menorrhagia and postpartum hemorrhage only when specific causes could be reasonably ruled out. Subjects complaining of epistaxis, whenever possible, were examined to exclude Rendu-Osler disease. A subject was recorded as definitely positive if at least two hemorrhagic symptoms were present. A family was recorded as positive if at least two members of the genetic line were positive. Laboratory investigation was offered to all available members of these families. The questionnaire

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answers and interviews of family members were examined by one of us before the laboratory results were known.

Laboratory investigations. Blood was taken by venipuncture with a 19-gauge needle in the infirmary of each school between 8:30 AM and 10:00 AM after the subjects had been resting for at least 30 minutes. The samples were placed in precoded tubes and transported in refrigerated boxes to our laboratory within two hours. Repeat blood samples were taken 2 to 4 months later from all children of hemorrhagic families, and samples were also obtained from as many family members as possible.

Blood cell counts were made on EDTA-anticoagulated blood using an electronic counter (Coulter Counter IV Plus, Coulter Electronics, Hialeah, FL). ABO blood groups and Rh typing were determined using standard slide methods; secretors were identified by a saliva test. Blood for coagulation tests, collected in 3.8% sodium citrate (1:10), was centrifuged at +4 °C (15 minutes at 1,500 g) immediately after arrival at our laboratory, and platelet-poor plasma was deep-frozen and stored at -80 °C.

The ristocetin cofactor activity of vWf was measured essentially according to the method of Macfarlane et al.¹⁷ The rate of agglutination of formalin-fixed washed platelets to ristocetin (final concentration, 1.5 mg/mL) was measured by calculating the slope of the steepest part of the agglutination curve (Chrono-Log Corp aggregometer, Havertown, PA). Four dilutions of local standard plasma in buffered saline (1:1 to 1:8) were assayed in reverse order, at the beginning and at the end of each set of measurements, and the reference curve was calculated on the average of the rate of agglutination of each dilution. No significant temporal drift occurred if the assay was completed within two hours. Each sample was assayed at three different dilutions and its potency in terms of the local standard plasma calculated by fitting a straight line parallel to the reference line. When major departures from parallelism were observed, the sample was retested. Two samples, one with normal and the other with low ristocetin cofactor activity, were included in each set of measurements; the interassay variability of both samples was less than 10%. The coagulant activity of factor VIII (VIII:C) was measured using a one-stage method based on the activated partial thromboplastin time (Actin reagent, Dade, Aguada, Puerto Rico) and a dilution of the test plasma in hemophilic plasma from patients with an unmeasurable VIII:C level.¹⁸ vWf antigen was measured by the Laurell electroimmunoassay technique using a polyclonal antiserum anti-vWf antigen from Behringwerke (Marburg, West Germany).¹⁹ The ratios of the potency of the samples to the local standard plasma were calculated as in the ristocetin cofactor assay.

The local standard plasma, plasma from 98 healthy hospital staff members, was collected on a single morning and pooled. The same local standard, stored at -80 °C, was used throughout the study. It was calibrated for factor VIII:C, vWf:Ag, and ristocetin cofactor activity against the First International Reference Preparation for Factor VIII Related Activities in Plasma (IRP) (kindly supplied by Dr T.W. Barrowcliffe, National Institute for Biological Standards and Control, London). Calibrations, three at the beginning and three at the end of the study, were made as suggested by Kirkwood and Snape.²⁰ The local plasma standard and the lyophilized IRP were diluted in plasma severely deficient in vWf for the purpose of calibrating ristocetin cofactor activity. The potency estimations were combined by taking a weighted geometric mean, the weights being inversely proportional to the variances of the log potency estimates. The ristocetin cofactor activity of vWf in plasma is simply referred to as "vWf" in the text. The multimeric composition of factor VIII/vWf was analyzed in the laboratory of Professor Mannucci (Milan, Italy), as previously published.²¹

Definition of the normal range of vWf. Values of vWf obtained from children without a family history of hemorrhage were used for

the definition of the normal range of vWf. An analysis of the variance and the Newman-Keuls test²² showed significantly lower level of vWf in blood group O subjects. Separate normal ranges were calculated for O and non-O subjects. The values were not normally distributed and showed significant positive kurtosis and skewness. Several transformations including log, square root, and inverse hyperbolic sine did not correct all kurtosis and skewness, and the Kolmogorov-Smirnov test²³ showed significant deviation from normality. Normal ranges were defined by calculating 2.5 and 97.5 percentiles by a nonparametric method.²⁴ A 90% confidence interval for each end point thus obtained was calculated on the basis of a binomial probability distribution.²⁴ All reference values were included because no outliers were found. The normal range of vWf in adults was defined in 289 subjects of both sexes aged 19 to 65 without a personal or family history of hemorrhage and who were not taking contraceptive drugs. One hundred twenty-four of them were regular blood donors, 98 were healthy hospital staff members, and 67 were teachers or employees of the schools.

Criteria for the diagnosis of vWd. The diagnosis was considered only for subjects belonging to a family recorded as positive for bleeding history. Subjects satisfying this criterion and also having vWf levels below the normal range were classified as cases with "probable vWd." A definite diagnosis of vWd was considered when at least one other member, on the hemorrhagic side of the subject's family, had a low vWf level.

RESULTS

The potency ratios of the local standard relative to the international standard measured at the beginning and at the end of the study 18 months later showed similar potencies. Potency estimations were combined and assigned to the local standard plasma (Table 1).

A total of 1,218 children participated in all stages of the research, with similar rates of participation in the areas north (594/620) and south (624/661) of Vicenza; the male-to-female ratio was 1.01. Among the questionnaires, 202 were selected because one or more of the reported episodes could be regarded as hemorrhagic. Parents of children of these families were interviewed. In this way, 49 families (21 north and 28 south of Vicenza) could be classified as positive (107 members with two or more hemorrhagic symptoms). Epistaxis accounted for 39% of the 268 identified symptoms, followed by bleeding after tooth extraction (20%), menorrhagia (18%), postpartum hemorrhage (8%), prolonged bleeding after superficial cuts (4%), after tonsillectomy or adenoidectomy (4.5%), after surgery (3.5%), easy bruising (2%), and other manifestations (1%). Values of vWf in the

Table 1. Potency Ratios of Local Standard Plasma Relative to the International Standard for Factor VIII/vWf-Related Activities

Test	At the Beginning of the Study (3 Calibrations)		At the End of the Study (3 Calibrations)		Combined Potency (6 Calibrations)	
	Mean	95% CL	Mean	95% CL	Mean	95% CL
vWf	1.07	0.97-1.16	1.08	0.96-1.19	1.07	1.01-1.13
VIII:C	0.93	0.78-1.07	0.86	0.76-0.94	0.89	0.83-0.94
vWf:Ag	1.11	0.94-1.31	1.03	0.89-1.19	1.06	0.97-1.15

Student's *t* test did not show statistically significant differences between potency ratio values found at the beginning and at the end of the study for each activity.

Abbreviation: CL, confidence limits.

children of these families (52 subjects, 24 male and 28 female) were treated separately and normal ranges calculated from the remaining 1,166 children.

Subjects of group O had a significantly lower mean vWf value than subjects of group A, B, or AB ($P < .001$; Newman-Keuls multicomparison test) (Table 2). The age distribution of vWf levels was independent of sex, Rh phenotype, secretory status, or area of residence. In 289 adults, vWf was also similarly lower in group O subjects, whereas no significant differences were observed in relation to sex or Rh phenotype. Only in subjects of group O was there a significant correlation with age ($r = .33$; $P < .001$). Table 2 shows the mean values and normal ranges of vWf for children and adults of O and non-O groups. A correction for age in group O adults was not done.

Figure 1 shows the distribution of vWf levels in the 52 children belonging to families with hemorrhagic histories.

Six subjects of group O and four of group non-O fell below the 2.5 percentile. Fourteen children had vWf levels below the upper limit of the 90% confidence limits (nine of group O, five of group non-O), and seven were below the lower limit (three of group O, four of group non-O). Subjects of group O in the normal range seem to be clustered near its lower end.

Table 3 lists the laboratory findings and hemorrhagic symptoms in the 14 children with low vWf levels and their symptomatic family members.

Six children (four to nine according to the 90% confidence interval of the 2.5 percentile) could be definitely classified as having vWd because other family members had vWf levels below the normal range, whereas for another four (three to five) the diagnosis of vWd was "probable." Similar prevalence of the disease was found in the two areas. vWd was more frequent in females (eight of 14) and in O group children (nine of 14), (P values, .091 and .105, not significant). vWf was below the normal range in nine of 24 relatives in whom it was measured (38%).

The strict correlation between vWf and vWf:Ag and between vWf and VIII:C (in both cases $r = .83$, $P < .001$) suggests that these subjects may have type I vWd. This was confirmed by multimeric analysis of all affected children. vWf levels in these subjects are distributed around half of the normal value, as would be expected in heterozygotes of an autosomal dominant trait. A clear hemorrhagic story could be traced in all families, and symptoms, present in all the children, appeared to be transmitted in an autosomal dominant fashion.

Table 2. Distribution of the vWf Level in Normal Subjects

Subjects	No.	Blood Group	vWf Level (IU/dL)		
			Mean (Range)	2.5 Percentile (90% CI)	97.5 Percentile (90% CI)
Children	510	O	99.7 (37.4-177.6)	61.0 (55.6-66.3)	149.8 (145.5-154.1)
	656	Non-O	115.8 (43.9-230.0)	77.0 (76.0-81.3)	164.0 (156.2-175.5)
Adults	121	O	88.7 (46.0-123.1)	51.8 (46.0-54.9)	118.8 (116.6-123.1)
	168	Non-O	110.6 (69.5-203.0)	75.1 (69.5-81.3)	167.6 (144.5-203.0)

Abbreviation: CI, confidence interval.

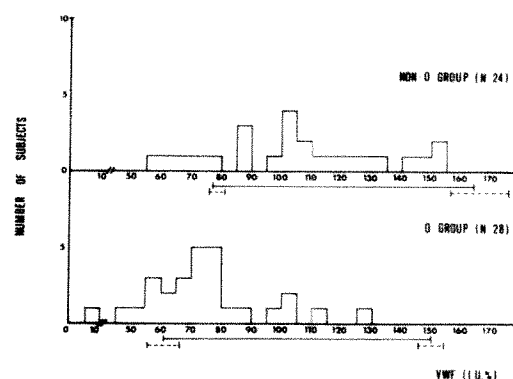


Fig 1. Distribution of vWf levels in the 52 children belonging to hemorrhagic families. Normal ranges (—) and 90% confidence intervals (---) around the lower and upper end points are indicated.

Repeat blood samples were obtained from 44 of the 52 children of hemorrhagic families 2 to 4 months after the first investigation. The mean values of vWf were the same as before (87.9 v 88.2), with a mean difference between first value and second values for each subject of 11.8 ± 9.5 . The vWf values of the second samples confirmed the diagnoses for 12 of the 14 children, and four additional cases of vWd were detected.

DISCUSSION

The criteria for the diagnosis of vWd used in this investigation were conservative and aimed at reducing to a minimum the rate of inclusion of false-positive subjects. Subjects belonging to hemorrhagic families that had vWf below the normal range were recorded as cases of "probable vWd." Definite diagnoses were made only when, in addition, at least one other member with low vWf could be found among relatives on the hemorrhagic side of the family.

Diagnostic criteria (laboratory and clinical). The lower normal limit of the vWf level was chosen as the value below which lie 2.5% of normal subjects and was defined on the basis of the values found in more than 1,000 normal subjects. Because the vWf distribution showed a positive kurtosis and skewness that was not completely removed by several transformations, we used a nonparametric method for the definition of the 2.5 and 97.5 percentiles and the 90% confidence intervals around these limits. Nonparametric methods make no assumption about the type of distribution of the reference values and are thus more precise and recommended in situations that differ from the ideal model of normality.²⁴⁻²⁶ Homogeneity between the reference and investigated groups was assured because both were drawn from the same population of children. Moreover, because vWf was found to be lower in group O subjects, separate normal ranges were calculated for O and non-O subjects. Uniformity of measurement of vWf during the study was guaranteed by using a single reference standard that showed no loss of potency relative to the international standard (Table 1) and by performing the vWf assay shortly after sample collection. No changes in vWf levels were found in relation to the time of blood sampling.

Table 3. Laboratory Findings and Hemorrhagic Symptoms in the Children Classified as Affected by vWd and Their Relatives

No.	Sex	Area	Blood Group	Children Under Investigation			Diagnosis	Bleeding Symptoms‡	Family Members		
				vWF	vWF:Ag (IU/dL)	VIII:C			Members	vWf (IU/dL)	Bleeding Symptoms‡
1	M	N	O	8*†	5	7	D	afg	Mother	21	abcdeg
									Sister	19	afg
									Uncle	ND	abg
2	F	S	O	50*†	38	50	D	ab	Mother	ND	abdefg
									Uncle	64	ab
									Brother	ND	a
									Grandmother	ND	ac
									Cousin	59	a
3	M	N	O	51*†	51	53	P	ag	Father	NV	ab
									Cousin	ND	ad
4	M	N	O	56†	53	37	D	a	Mother	48	acg
									Sister (see case 7)		
									Grandmother	NV	ac
5	F	S	O	56†	63	40	P	abfg	Mother	NV	abf
									Brother	NV	a
6	F	N	O	58†	53	54	D	ac	Mother	NV	a
									Sister	48	ac
									Aunt	53	ad
7	F	N	O	64	50	46	D	a	Sister of case 4		
8	M	N	O	64	53	57	D	ag	Mother	NV	acdeg
									Brother	53	a
									Aunt	ND	ac
9	F	N	O	64	36	36	D	a	Father	NV	a
									Brother	49	ab
									Brother	NV	a
									Aunt	ND	ac
10	F	S	A	59*†	54	57	D	a	Father (uncle, case 2)		
									Cousin (see case 2)		
									Aunt (mother, case 2)		
11	F	S	A	62*†	68	56	P	acg	Mother	NV	bc
12	F	S	A	67*†	54	60	P	ab	Mother	NV	ab
13	F	S	A	67*†	50	64	D	c	Sister	56	ac
									Mother	NV	acg
									Aunt	ND	e
									Cousin	NV	a
14	F	S	B	78	72	61	P	a	Mother	NV	abce
									Sister	NV	a
									Brother	ND	ad

VIII:C and vWf:Ag determined in 60 normal children ranged 56 to 182 and 53 to 188 respectively.

Abbreviations: ND, not done; NV, normal value.

*Below the lower limit of the 90% confidence interval of 2.5 percentile.

†Below the 2.5 percentile.

‡a, epistaxis; b, bleeding after tooth extraction; c, menorrhagia; d, bleeding after surgical procedures; e, postpartum hemorrhage; f, prolonged bleeding after superficial cuts; g, easy bruising (considered for the purpose of defining hemorrhagic diathesis only in male adults).

Conservative criteria were used for the definition of hemorrhagic diathesis, and two or more positive members were required to consider a family as positive. The frequency of "bleeders" in the general population is unknown, but using criteria less strict than the ones adopted in this study, Miller et al²⁷ were able to classify as symptomatic as many as 23% of normal subjects, a figure that would undermine the discriminant power of this parameter. In contrast to their data, only 26 children (2%) and only 107 relatives among the approximate 14,000 for whom we had direct or indirect information through questionnaires and interviews were classified as positive. In this way, a total of 52 subjects (from 49 families) out of 1,218 investigated were recorded as belonging to hemorrhagic families.

The a priori probability of a child being a member of a hemorrhagic family is thus .042 (52/1218), which, when multiplied by .025 (the probability of false-positive classification on the basis of vWf measurement), yields a probability of about .001 of a "normal child" being falsely classified as a case of "probable vWd." The probability of misclassification is, of course, greatly reduced in the presence of other relatives with low vWf levels, and in this case it seems appropriate to consider the diagnoses as definite.

Epidemiology of vWd. Among the 1,218 children investigated, ten were classified as affected (four cases as "probable vWd," six cases as definitely proven). More precisely, taking into consideration the 90% confidence interval of the lower end point of the normal range (in fact, all the values in

this interval have identical significance), there would be from seven (three "probable cases," four definite cases) to 14 (five "probable cases," nine definite cases). The calculated prevalence of vWd in our population is thus estimated to be between 0.57% and 1.15% (Table 3). All these subjects appear to be heterozygous and to have inherited the disorder in an autosomal dominant fashion. The phenotypic and laboratory characteristics and multimeric composition were those of classic vWd (type I).

A theoretical prevalence of 25 of 1,000,000 homozygotes is anticipated according to the Hardy-Weinberg equation on the basis of 1% heterozygotes in the population, but a possible genotypic heterogeneity of classic vWd, unrecognized by our methodology, would make the Hardy-Weinberg equation²⁸ inapplicable. Nevertheless, homozygous patients for the classic type I dominant vWd are likely to be more frequent than homozygotes for type III recessive vWd, who are estimated to be between 0.11 and 0.55 out of 1,000,000 in Europe²⁹ and 3.1 and 3.2 out of 1,000,000 in Sweden and Israel.^{14,29,30} Thus, in diagnosing type III vWd, one has to be very careful that the proband is not the offspring of two nonmanifesting type I patients because rare instances are known in which severely affected cases were born of asymptomatic type I vWd parents.^{27,31}

Although repeated measurements of vWf in patients with vWd may yield variable results,⁷ the classification of cases in this investigation showed little dependence on repeating the vWf assay. In fact, 12 of the 14 subjects had their diagnoses confirmed by the second value. Moreover, four additional children would have been detected by considering the lowest value found in the two determinations.

An assay for vWf (ristocetin cofactor activity) was found to be the single most useful test for identification of transmitters and symptomatic members in kindreds with the disease.^{32,33} In this study a vWf assay was preferred to other tests such as a vWf antigen (vWf:Ag) on the basis of this consideration and because variant forms of vWd are known in which vWf:Ag is normal or near normal. Nevertheless, Miller et al³² could identify only 42% of the transmitters by the vWf assay in two large kindreds with classic vWd. In several large kindreds with classic vWd by using separate normal ranges for O and non-O subjects, we were able to identify about 50% of the transmitters (data not published), and in reasonable accord with these figures, in this study we were able to identify as transmitters about 40% of the hemorrhagic relatives in families of children with low vWf.

Considering the limitations of the vWf assay for diagnosis and the conservative criteria for the definition of hemorrhagic diathesis, probably 50% or more of vWd cases could go unrecognized with a methodology like the one used in this study (false-negative subjects). Nevertheless, the prevalence of vWd found in this investigation is much higher than the prevalence previously estimated from cases registered at

specialized centers, which appeared to be similar to or even lower¹³⁻¹⁶ than that of hemophilia.³⁴ This method of calculating the prevalence of a disease with highly variable clinical expression is unreliable. Moreover, the significantly lower level of ristocetin cofactor activity (this study) and vWf antigen in subjects of group O^{35,36} leads to an overestimation of vWd in O subjects and to an underestimation in non-O subjects unless strictly defined normal ranges are used.

Although the two geographic areas investigated here can be regarded as independent samples, almost identical prevalences of vWd were found. Hence, because these areas do not present particular features, we think that the prevalence of vWd estimated in this study can apply to the Caucasian population in general. However, in extrapolating the prevalence found in children aged 11 to 14 to subjects of all ages we must consider a possible bias in the upward direction. In fact, children from larger families have a greater probability of being included, and thus there is a potential "founder effect." Actually, only one family among those who contributed with positive cases had additional children (either positive or negative) included in the study. Thus, although subjects 4 and 7 in Table 3, who are siblings, should be considered as a single case, this does not substantially change our prevalence estimation.

Practical implications. Assuming a 2% prevalence of subjects carrying the mutant gene for vWd in the population and a 50% sensitivity of the vWf assay in identifying these carriers, the vWf assay would have a predictive value of 29% for positive results (true positives/total positives) and 98.9% for negative results (true negatives/total negatives). The efficiency of the test (percentage of true-positive and true-negative results) would be 96.5%. Furthermore, limiting its use for subjects with evidence of personal or family histories of hemorrhage would virtually abolish the misclassification of normal subjects (about one in 1,000 false-positives in this study). It has to be emphasized that, to obtain these results, normal ranges have to be defined separately for O and non-O subjects.

Considering the frequency of vWd suggested by this study, we think that a vWf assay (ristocetin cofactor activity) should be included among the first level screening tests for evaluation of subjects with mild bleeding diathesis even in presence of normal routine screening tests.

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Fibrinolysis in Pregnancy: A Study of Plasminogen Activator Inhibitors

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During pregnancy the plasma concentration of two different inhibitors of plasminogen activators (PAIs) increases. The only one found in the plasma of nonpregnant women (PAI1) is immunologically related to a PAI of endothelial cells; its plasma activity, as deduced from the inhibition of single-chain tissue-type plasminogen activator (t-PA), increased from 3.4 ± 2.3 U/mL (mean $\pm$ 95% confidence limits) in the plasma of nonpregnant women to 29 ± 7 U/mL at term, and its antigen level, measured by a radioimmunoassay, increased from 54 ± 17 ng/mL to 144 ± 25 ng/mL. In pregnancy plasma a second PAI (PAI 2) related to a PAI found in placenta extracts was observed. Its level, quantified with a radioimmunoassay, increased from below the detection limit (~ 10 ng/mL) in normal plasma to 260 ng/mL at term. One hour after delivery, PAI 1 activities and antigen decreased sharply, but the PAI 2 antigen levels remained constant. Three days later, the PAI 1 antigen levels had fallen to normal levels, but the PAI 2

antigen levels were still at least eightfold above the nonpregnant values. During pregnancy, the t-PA and pro-urokinase (u-PA) antigen concentrations increased 50% and 200%, respectively, whereas the plasminogen and α_2 -antiplasmin levels remained constant. Despite the large variations in the levels of PAs and PAIs, the overall fibrinolytic activity as measured in diluted plasma by a radioiodinated fibrin plate assay did not change significantly. Just after delivery, a great increase in the t-PA antigen levels was observed. Three to five days after delivery most parameters of the fibrinolytic system were normal again. Our results demonstrate that during pregnancy and in the puerperium profound alterations of the fibrinolytic system occur that are characterized by increases in PAs and their inhibitors, but these alterations do not affect the overall fibrinolytic activity.

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DURING PREGNANCY profound changes in the hemostatic system are observed. Plasma levels of fibrinogen, factor VII, factor VIII and von Willebrand's factor increase.¹⁻⁴ The prevailing opinion states that the fibrinolytic activity of plasma, as measured in euglobulin precipitates, decreases during pregnancy.⁴⁻¹² Some authors, however, observed an increased fibrinolytic activity of unfractionated whole blood¹³ and a higher level of fibrin degradation products.¹⁴ The decreased fibrinolytic activity of euglobulins is generally attributed to an increased plasma level of a plasminogen activator inhibitor (PAI), possibly derived from the placenta.^{10-12, 15-17} These studies, however, did not take into account the existence of at least two different PAIs, one purified from endothelial cells (PAI 1)¹⁸ and one purified from placenta,¹⁹ leukocytes,²⁰ and histiocytic lymphoma cells (PAI 2).²¹ In a recent study, Nilsson et al showed that both PAIs are present in pregnancy plasma.²² No study exists yet that separately measures the levels of the two PAIs. With the development of more specific methods to determine the two PAIs, tissue-type plasminogen activator (t-PA), and urokinase (u-PA) in human plasma it is now possible to analyze in more detail the modifications of the

fibrinolytic system during pregnancy. In the present study we therefore measured in nonpregnant women, during pregnancy and after delivery, the plasma antigen levels of PAI 1, PAI 2, t-PA, u-PA, plasminogen and α_2 -antiplasmin, and also the PA and PAI 1 activity. Our results show that during pregnancy the levels of the two PAIs, t-PA, and u-PA strongly increase but that the overall fibrinolytic activity remains relatively constant.

MATERIALS AND METHODS

Materials. Thrombin (Topostasin) was obtained from Hoffmann-La Roche, Basel, Switzerland; fibrinogen (bovine, plasminogen rich) from Poviet, Oss, The Netherlands; ¹²⁵I-fibrinogen (FIBI-125C, 1 mCi/mg) from Sorin, Saluggia, Italy; aprotinin (Trasylol) from Bayer, Leverkusen, Federal Republic of Germany; lodogen from Pierce, Oud Beijerland, The Netherlands; Na¹²⁵I from New England Nuclear, Dreieich, Federal Republic of Germany; all reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad, Richmond, CA; and Sephadex G-100, Concanavalin A (ConA) Sepharose, CNBr-activated Sepharose, lysine-Sepharose, and protein A-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden. The World Health Organization International Reference Preparations of human u-PA (NIBSC 66/46) and human t-PA (NIBSC 83/517) were provided by Dr P.J. Gaffney from the National Institute for Biological Standards and Control, London.

Plasminogen activators. Human t-PA was purified as previously described. It consisted mainly of single-chain (sc) t-PA.²³ u-PA (two-chain [tc], 54 kDa) was a gift of Dr Sauser of Sero Laboratories, Coinsins, Switzerland. Antisera against t-PA and u-PA were raised in rabbits. IgG was isolated by affinity chromatography on protein A-Sepharose. u-PA was radioiodinated with carrier-free ¹²⁵I by using lodogen according to the procedure of Fraker and Speck,²⁴ as described before.²⁵ In general, 20% to 40% of added ¹²⁵I was incorporated into protein under the labeling conditions used. The radioiodinated u-PA had a specific radioactivity of 10 to 40 nCi/ng (between 10 and 40 atoms of ¹²⁵I incorporated per 100 molecules of u-PA). The activity of the t-PA and u-PA preparations used in this study were calibrated against their respective international reference preparations in a fibrin plate assay.

Plasminogen activator inhibitors. PAI 1 was purified from the

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conditioned medium of hydrocortisone (10^{-7} mol/L) stimulated cells of the human fibrosarcoma cell line HT 1080²⁶ by affinity chromatography on ConA-Sepharose,¹⁸ ion-exchange chromatography on diethylaminoethyl Sephacel, and gel filtration on Sephadex G-100. The final product showed one band on reduced and nonreduced SDS-PAGE and was immunoprecipitable by antibodies prepared against a PAI purified from the conditioned medium of bovine¹⁸ and human²⁷ endothelial cells (gifts from Drs Loskutoff, La Jolla, CA, and Sprengers, Leiden, The Netherlands) respectively.

PAI 2 was purified from the conditioned medium of phorbol myristate acetate (30 ng/mL)-stimulated cells of the human histiocytic lymphoma cell line U-937, and anti-PAI 2 antibodies were raised in rabbits as previously described.²¹ PAI 1 did not react with antibodies to PAI 2 and vice versa. PAI 2 was found to be indistinguishable from a PAI partially purified from human placenta by the following criteria: (1) a line of complete identity was obtained in double immunodiffusion, (2) identical molecular weights were obtained after SDS-PAGE followed by electroblotting and immunologic detection using immunopurified anti-PAI 2 IgG and peroxidase-conjugated goat-IgG antirabbit IgG, and (3) the second-order rate constant of the reaction of placental PAI and PAI 2 with u-PA and t-PA were similar.²¹ PAI 1 and PAI 2 were radioiodinated using the method of Fraker and Speck.²⁴ Radioiodinated inhibitor was separated from free ¹²⁵I by passage over a 12-mL column of Sephadex G-25. The specific radioactivity of the radiolabeled PAI preparations was between 15 and 50 nCi/ng (corresponding to 15 to 50 molecules of ¹²⁵I incorporated per 100 molecules of PAI 1 or PAI 2).

Plasmas. Blood was obtained by venipuncture, anticoagulated with buffered citrate (nine parts of blood were mixed with one part of 0.1 mol/L sodium citrate/citric acid, pH 4.5), and centrifuged for 15 minutes at 3,600 g at 4 °C. The plasmas were stored at -70 °C. A normal plasma pool was made from the blood of 50 healthy blood donors.

Subjects. The subjects studied were (1) 90 pregnant women whose pregnancy proceeded normally. For each 4-week period of pregnancy, except the first, ten women were admitted. The last period comprised the 37th week until term. Also studied were (2) ten women postpartum (mean time since delivery, 77 ± 54 minutes [mean $\pm$ SD]), (3) ten women three to five days after delivery, and (4) ten apparently healthy, nonpregnant women of the same age group (27 ± 4 years) as the pregnant women (27 ± 5 years).

Immunoradiometric assay for t-PA in plasma. The t-PA antigen levels in human plasma were determined by using an immunoradiometric assay essentially as described by Rijken et al.²⁸ Standard curves were made using a t-PA preparation standardized against the International References Preparation of t-PA. This preparation has a specific activity of 500,000 IU/mg protein (1 IU = 2 ng).

Radioimmunoassay for (pro) u-PA in plasma. One hundred microliters of plasma diluted five times with immunodilution buffer (50 mmol/L Tris-HCl, pH 7.4, 200 mmol/L NaCl, 10 mmol/L EDTA, 0.1% bovine serum albumin, and 0.05% Na₂S₂O₃) was mixed with 100 μ L anti-u-PA IgG that had been diluted such (1:100,000) that in the absence of added cold u-PA half of the ¹²⁵I-labeled u-PA bound to the antibodies. The sample was incubated four hours at room temperature with IgG. Thereafter, 50 μ L of ¹²⁵I-u-PA (containing 2,000 cpm radiolabel) was added and incubated overnight at 4 °C. The following day, 100 μ L of a cellulose suspension to which donkey antibodies to rabbit IgG had been coupled (Sac-Cel, Wellcome Reagents, Beckenham, UK) was added. The suspension was incubated 30 minutes at room temperature, diluted by the addition of 2 mL of 0.15 mol/L NaCl, and centrifuged for five minutes at 200 g. The pellet was twice washed with 2 mL of 0.15 mol/L NaCl and counted in a gamma counter. The radioactivities after deduction of

the nonspecific binding of ¹²⁵I-u-PA to Sac-Cel in the absence of added IgG were compared in a logit-log plot with a standard curve of u-PA that had been calibrated against the international reference preparation of u-PA (specific activity, 100,000 U/mg).

Radioimmunoassay for PAI 1 and PAI 2 in plasma. The methodology used for the determination of PAI 1 and PAI 2 antigen was identical to that described for u-PA. Rabbit antbovine PAI 1 antiserum (a gift from Dr Loskutoff, La Jolla, CA) was used in a dilution of 1:1,000, and immunopurified rabbit anti-PAI 2 IgG was used in the concentration of 60 ng/mL. The antigen levels were measured by comparison in a logit-log plot with standard curves of pure PAI 1 or PAI 2. The protein concentrations of the PAI 1 or PAI 2 stock solutions were determined using the protein assay of Bradford²⁹ and bovine serum albumin as the standard.

Assay for plasminogen and α_2 -antiplasmin. The plasminogen and α_2 -antiplasmin levels were determined by rocket immunoelectrophoresis using commercially available antisera (Behringwerke, Marburg an der Lahn, Federal Republic of Germany). Rocket heights were compared with those of various dilutions of a normal human plasma pool and the results expressed in percent with respect to the plasma pool.

Assay for the total PA activity in plasma. The PA activity was measured using a ¹²⁵I-fibrin plate method.^{30,31} Plasminogen-free bovine fibrinogen was converted to fibrin monomers and further purified by three steps of polymerization and dissolution in acetic acid as described by Haverkate and Timan.³² Human ¹²⁵I-fibrinogen of clinical grade was rendered plasminogen free by affinity adsorption to lysine-Sepharose.³³ The ¹²⁵I-fibrin plate was prepared using these two fibrinogen solutions exactly as described by Sharoni et al.³¹ No thrombin was detectable in these plates with a chromogenic substrate assay (detection limit of 5 pg/mL). Prior to use, the wells were incubated for 15 minutes with sterile phosphate-buffered saline. The buffer was replaced with a mixture of 250 μ L of a solution of plasminogen (5 μ g/mL) and 100 μ L of a 50- or 100-fold dilution of plasma in 0.1 mol/L Tris-HCl, pH 7.5, containing 1% bovine serum albumin. The plates were incubated at 37 °C; after three and six hours, 50- μ L aliquots were removed to determine the degree of ¹²⁵I-fibrinolysis. The percentage of ¹²⁵I-fibrinolysis was compared with that of standard curves using a laboratory reference preparation of human t-PA that had been calibrated against the international t-PA reference preparation.

PAI 1 activity assay. Because the reaction rate between PAI 2 and sc t-PA is quite slow^{21,34} but fast between PAI 1 and sc t-PA,²⁵ we have adapted the previously described assay for PAI activity in plasma,²⁵ taking care to add to the plasma a t-PA preparation (final concentration, 42.5 U/mL) that contained less than 10% of the tc form.

Preparation of immobilized antibodies. IgG was prepared by affinity chromatography on protein A-Sepharose from 0.5 mL of rabbit preimmune serum, antiserum directed against PAI 1,¹⁸ and antiserum directed against PAI 2.²¹ The IgG was dialyzed overnight against two changes of 0.1 mol/L sodium bicarbonate, pH 8.1, and coupled to 1 mL of CNBr-activated Sepharose 4B.

Immunologic comparison of PAIs in normal and pregnancy plasma. Radioiodinated u-PA (10 μ L, 200 ng/mL, 4×10^6 cpm/mL) was incubated for ten minutes at 37 °C with 200 μ L of human plasma. Forty microliters of the mixture was added to 200 μ L of a 25% suspension of immobilized preimmune IgG, anti-PAI 1 or anti-PAI 2 IgG in 50 mmol/L imidazole and 140 mmol/L NaCl, pH 7.35. After a further 20 minutes' incubation at 25 °C, the gel was spun down (five minutes, 1,000 g) and the supernatant diluted twofold with SDS sample buffer. SDS-PAGE in a 7.5% gel³⁵ of a 50- μ L sample and autoradiography was performed as described previously.²⁵

RESULTS

PAI 1 and PAI 2 levels in pregnancy plasma. The PAI 1 activity in the plasma of nonpregnant women was extremely variable (3.4 ± 2.3 U/mL, mean $\pm$ 95% confidence limits of the error of the mean). In the first 3 months of pregnancy, the PAI 1 activity was slightly, but nonsignificantly higher. Thereafter an almost linear increase in the PAI 1 activity to 29 ± 7 U/mL at term was observed (Fig 1). One hour after delivery, the PAI 1 activity decreased approximately 50% to 15 ± 9 U/mL, and the levels were normal three to five days after delivery.

As observed for the PAI 1 activity, the PAI 1 antigen levels in nonpregnant women were quite variable (55 ± 17 ng/mL). During the first 2 months of pregnancy, the PAI 1 antigen levels appeared to decrease slightly; thereafter they increased steadily to a maximum of 147 ± 32 ng/mL, attained between 32 and 36 weeks of pregnancy (Fig 2). After delivery the PAI 1 antigen levels decreased sharply within one hour and were normal three to five days later.

The PAI 2 antigen levels in nonpregnant women were below the detection limit (<10 ng/mL). From 4 to 8 weeks of pregnancy the PAI 2 was measurable in about half of the subjects. After that it was detectable in all plasmas, and an almost linear increase of PAI 2 antigen concentration was observed until the 32nd week of pregnancy when the mean levels stabilized at around 240 ng/mL (Fig 3). In contrast to the PAI 1 activity and antigen, the PAI 2 antigen did not decrease within one hour postpartum. Three to five days after delivery the mean level of 77 ± 27 ng/mL of PAI 2 was still at least eightfold higher than in nonpregnant women.

Complex formation of u-PA with PAI 1 and PAI 2 in normal and pregnancy plasma. To verify whether PAIs

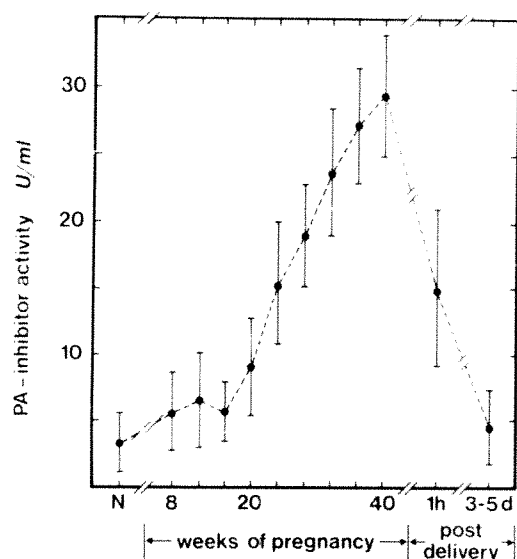


Fig 1. PAI 1 activity in plasma during pregnancy and postdelivery. The PAI 1 activity in plasma was estimated from the inhibition of sc t-PA, as indicated in Materials and Methods. Plasmas were obtained from ten nonpregnant (N) and 90 pregnant women. Each 4-week period is represented by ten women. Plasmas were also obtained from ten women one hour postpartum and ten women three to five days after delivery. The results are expressed as the means $\pm$ 95% confidence limits of the error of the mean.

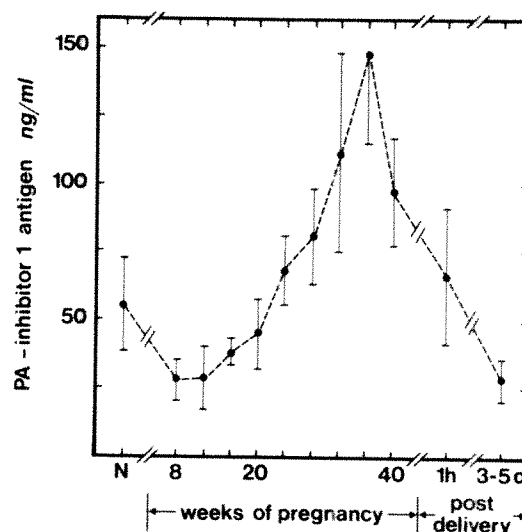


Fig 2. Plasma antigen levels of PAI 1 during pregnancy and postdelivery. The PAI 1 antigen levels were measured by a radioimmunoassay in the plasma of the 120 women indicated in the legends to Fig 1.

other than PAI 1 and PAI 2 are present in normal and in pregnancy plasma, ^{125} I-u-PA was added to a plasma pool obtained from nonpregnant women or from women in the last trimester of pregnancy and the immunologic identity of the complexes formed established. For this experiment u-PA was chosen rather than t-PA because u-PA reacts rapidly with both PAIs and sc t-PA only with PAI 1. The incubation of ^{125} I-u-PA in normal plasma resulted mainly in the formation of a 95 kDa complex (Fig 4). This complex bound to immobilized anti-PAI 1 IgG but not to anti-PAI 2 IgG. In pregnancy plasma, three complexes in the molecular weight region of 85,000 to 110,000 were formed. The largest and the smallest ones were retained by immobilized anti-PAI 2 IgG and the middle one by anti-PAI 1 IgG. These results,

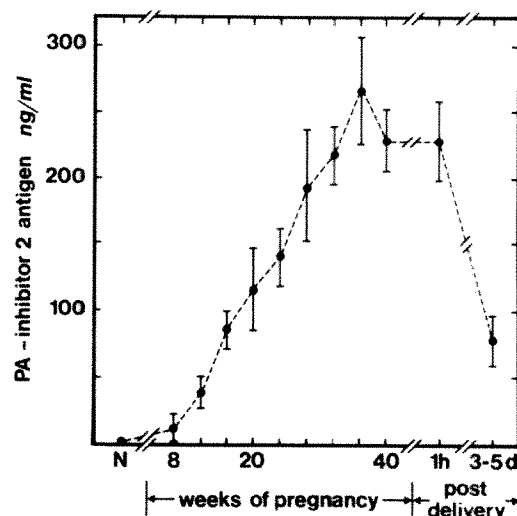
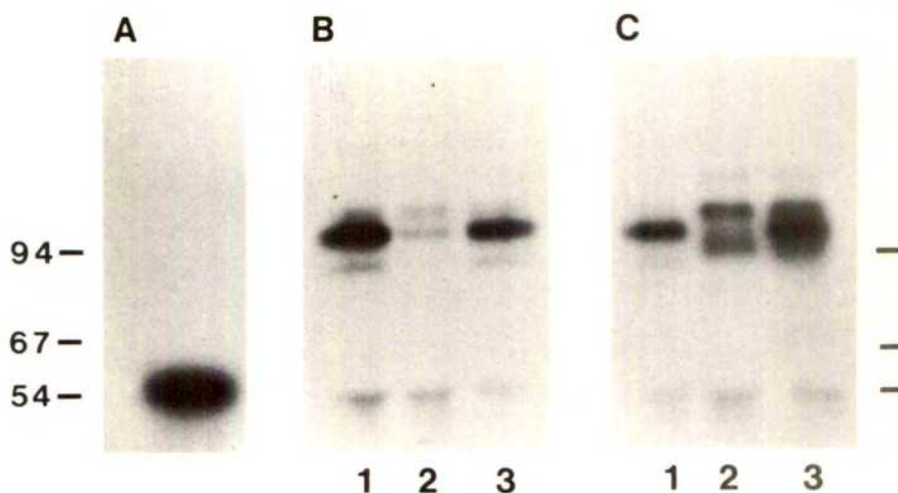


Fig 3. Plasma antigen levels of PAI 2 during pregnancy and postdelivery. The PAI 2 antigen levels were measured by a radioimmunoassay in the plasma of the 120 women indicated in the legends to Fig 1.

Fig 4. Immunologic identity of inhibitors of u-PA in normal plasma and late pregnancy plasma. (A) ^{125} I-u-PA alone. (B and C) ^{125} I-u-PA incubated at a final concentration of 20 ng/mL for ten minutes in a plasma pool of nonpregnant (B) or late pregnancy women (C). Thereafter the plasmas were passed either over a column of immobilized anti-PAI 2 IgG (lane 1), anti-PAI 1 IgG (lane 2) or preimmune IgG (lane 3). The complexes not bound to the columns were analyzed by SDS-PAGE in a 7.5% gel followed by autoradiography.



therefore, provide evidence that in pregnancy plasma PAI 1 exists in one form, whereas two different forms of PAI 2 might exist.

PA activity in plasma during pregnancy. Figure 5 shows that the PA activity in nonfractionated diluted plasma as measured on radioactive fibrin plates remained essentially constant during pregnancy and shortly after delivery. The slight changes observed were not significant. However, three to five days after delivery the PA activity levels were significantly lower than during the last period of pregnancy ($P < .001$, Mann-Whitney test) or in nonpregnant control women ($P < .005$).

Antigen levels of t-PA, (pro)-u-PA, plasminogen and α_2 -antiplasmin in pregnancy plasma. The surprising observation of an unchanged fibrinolytic activity in the presence of strongly increased levels of two different PAIs prompted us to study also the t-PA, (pro)-u-PA, plasminogen, and α_2 -antiplasmin antigen levels. During pregnancy the t-PA antigen increased from 2.2 ± 0.8 ng/mL (mean $\pm$ 95% confidence limits) in nonpregnant women to a plateau value between 3.0 and 3.6 ng/mL in the last two trimesters of

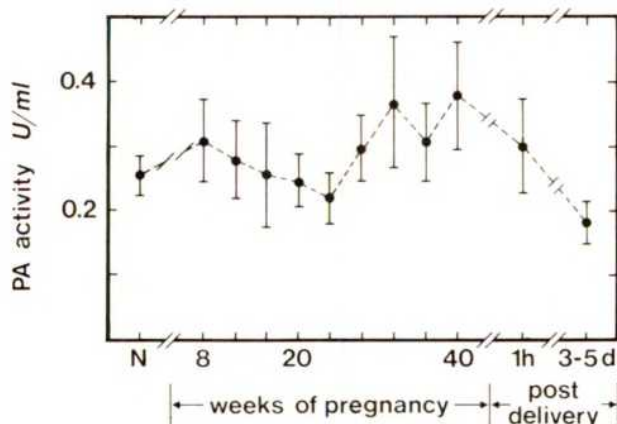


Fig 5. Plasma PA activity during pregnancy and postdelivery. The PA activity was measured on a radioactive fibrin plate using diluted nonfractionated plasmas of the 120 women as indicated in the legend to Fig 1.

pregnancy (Fig 6). One hour after delivery a doubling of the t-PA levels (6.5 ± 1.1 ng/mL) with respect to late pregnancy values was observed. Three to five days after delivery they were slightly, but significantly higher than those in nonpregnant women. The behavior of (pro)-u-PA antigen during pregnancy was quite different. An almost linear increase from 19 ± 3 ng/mL in nonpregnant women to a plateau level of 46 ± 3 ng/mL at 32 weeks of pregnancy was observed (Fig 7). Thereafter the mean (pro)-u-PA levels remained near this value until delivery. Just after delivery the u-PA levels increased slightly, but significantly ($P < .05$) to 52 ± 4 ng/mL. Three to five days postpartum the mean u-PA level was still greatly increased with respect to that in nonpregnant women (35 ± 3 v 19 ± 3 ng/mL; $P < .01$).

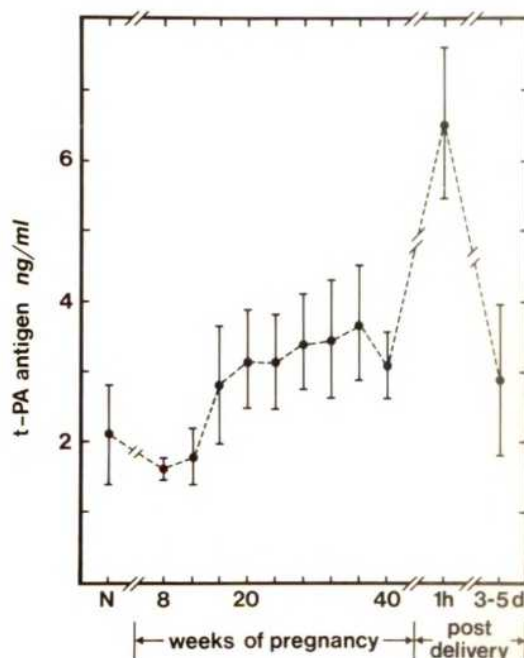


Fig 6. t-PA antigen levels during pregnancy and postdelivery. The t-PA antigen levels were measured by an immunoradiometric assay in the plasma of the 120 women as indicated in the legend to Fig 1.

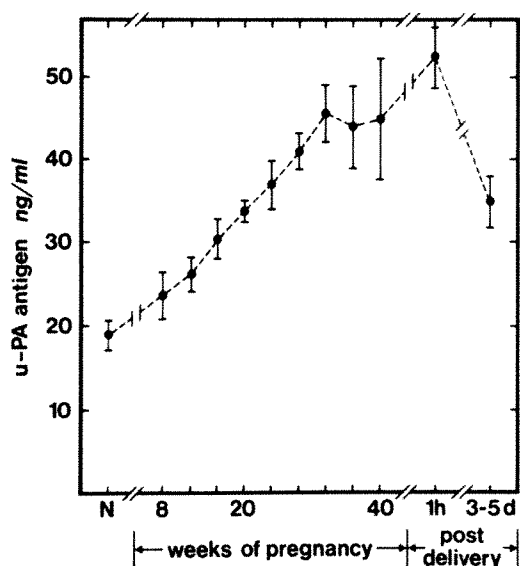


Fig 7. (Pro)-u-PA antigen levels during pregnancy and postdelivery. The u-PA antigen levels were measured by a radioimmunoassay in the plasma of the 120 women as indicated in the legend to Fig 1.

A slight but not significant increase in the plasminogen and α_2 -antiplasmin levels was observed during pregnancy and in the puerperium.

DISCUSSION

It has been known for many years that during pregnancy profound modifications of the coagulation and the fibrinolytic system occur.¹⁻¹⁷ Our study, however, is the first to measure systematically and quantitatively the behavior of several newly described fibrinolytic components of human plasma, particularly of two different inhibitors of t-PA and of u-PA. PAI 1 is synthesized by the endothelial cells and is present in plasma and in blood platelets.^{18,25,27,36-38} PAI 1 exists in an active and a latent form.^{39,40} The active form of PAI 1 readily inhibits tc u-PA³⁶ and tc t-PA and also sc t-PA²⁵ but is rather labile and at room temperature is transformed to a latent (inactive) form with a half-life of approximately two hours.⁴⁰ Antibodies directed against PAI 1 do not cross-react with PAI 2 produced by histiocytic lymphoma cells and vice versa.^{21,22} PAI 2 also has been extracted and purified from human placenta and is therefore often termed placental PAI.^{15,19} It is a very efficient inhibitor of tc u-PA and tc t-PA but inhibits sc t-PA poorly.^{25,34}

We observed striking increases in the level of PAI 1 and PAI 2 as well as moderate increases in the t-PA and u-PA antigen levels. Our quantitative measurements confirm the recent qualitative observations of Nilsson et al who found that pregnancy plasma contains two different PAIs²² and those of Lecander and Åstedt⁴¹ who reported increased antigen levels of a placenta-derived PAI with characteristics similar to that of PAI 2. The PAI 1 levels increased steadily after the 20th week of pregnancy and, at term, were threefold higher than in nonpregnant women. The PAI 2 levels were below the detection limit in normal plasma and, at term,

were more than 25-fold increased. The PAI 1 activity levels in plasma, as determined from the inhibition of sc t-PA, correlated better with PAI 1 than with PAI 2 antigen concentration. This was particularly noteworthy just after delivery when the PAI activity and PAI 1 antigen levels decreased sharply and PAI 2 remained high. We may therefore conclude that the PAI 1 activity assay is relatively insensitive to PAI 2. We observed, however, a quantitative discrepancy between the PAI 1 activity and PAI 1 antigen levels. In the last trimester, the PAI activity was on average 28 U/mL, corresponding to 60 ng of PAI per milliliter of plasma, whereas the PAI 1 antigen levels were approximately twofold higher. Two possible explanations for this discrepancy exist. In normal plasma virtually all t-PA is bound to PAI 1⁴²; consequently, about 3 to 4 ng/mL of PAI are bound to t-PA and not measured by our activity assay. However, because the observed discrepancy is one order of magnitude greater, it can only partially be explained by the inactivation of PAI 1 by t-PA. We must therefore assume that part of the plasmatic PAI 1 is present in the latent, inactive form.^{27,39,40}

When added to pregnancy plasma, tc u-PA formed two different complexes with PAI 2 of approximately 95 and 115 kDa. The two forms of PAI 2 probably correspond to a heavier glycosylated and a lighter nonglycosylated form of PAI 2⁴³ or may be due to cleavage of the PAI 2 molecule after its interaction with u-PA.²¹

Within one hour after delivery, the concentration of some components of the fibrinolytic system changed dramatically: the doubling of t-PA levels is probably due to release from the vessel walls induced by the stress of childbirth and may be responsible for the drop of the PAI 1 activity and antigen levels to about half the levels found at term. No differences were observed for u-PA and PAI 2 levels and for PA activity. Three to five days after delivery the PAI activity and t-PA and PAI 1 antigen levels were not significantly different from control values, but the u-PA and PAI 2 levels were still significantly elevated, whereas the PA activity was significantly decreased. Because u-PA half-lives are relatively short,⁴⁴ we have to assume that the increased production and release and/or the decreased clearance of u-PA persisted for several days after delivery. In previous work the rapid decline in PAI activity after delivery was explained by the arrest of PAI release from placental tissue into the circulation after the expulsion of the placenta.⁹ The persistence of PAI 2 suggests either that its main site of synthesis in pregnancy and after delivery is not the placenta or that its in vivo half-life is on the order of several days. In contrast, the rapid decrease in PAI activity and PAI 1 antigen levels after delivery might suggest that PAI 1 is produced by the placenta and that it has a short in vivo half-life. The latter is in accordance with the observations of Colucci et al on the short PAI half-life in rabbits.⁴⁵

We found that the PA activity remained essentially constant during pregnancy but was slightly decreased three to five days after delivery. Our results differ from those of Arias et al¹³ who observed a significant increase in the whole blood fibrinolytic activity in pregnancy and post partum and from most other studies reporting a significant decrease in the

fibrinolytic activity of euglobulins.⁴⁻¹² Measurement of the fibrinolytic activity in euglobulin precipitates, however, has several drawbacks: (1) u-PA precipitates only partially in the euglobulins; (2) during pregnancy, the fibrinogen levels increase almost twofold and thus prolong the euglobulin clot lysis time; and (3) PAIs precipitate partially in the euglobulins (unpublished observations) and thereby affect the fibrinolytic assay. It is therefore evident that the results of fibrinolytic activity measurements in pregnancy are critically dependent on methodology, particularly on whether the activity is measured in whole blood, plasma, or euglobulins. The increased levels of fibrin degradation products during

pregnancy measured by Fletcher et al¹⁴ indicate that the +ibrinolytic system remains functionally active and do not support the hypothesis that its activity decreases significantly during pregnancy.

Our study demonstrates that the changes in the fibrinolytic system during pregnancy are much more complex than hitherto assumed. The physiological meaning of and the mechanisms inducing these changes are at present obscure.

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Effect of Recombinant Human Tumor Necrosis Factor on the Colony Growth of Human Leukemia Progenitor Cells and Normal Hematopoietic Progenitor Cells

By Takuhei Murase, Tomomitsu Hotta, Hidehiko Saito, and Ryuzo Ohno

The effects of recombinant human tumor necrosis factor (rH-TNF) on the colony growth of human leukemia progenitor cells (L-CFU), granulocyte-macrophage progenitor cells (CFU-GM), and erythroid progenitor cells (BFU-E) were studied. L-CFU was assayed with leukemia cells obtained from patients with acute myelogenous leukemia. CFU-GM and BFU-E were assayed with bone marrow cells obtained from hematologically normal donors and patients with acute leukemia or non-Hodgkin's lymphoma in complete remission. A dose-dependent growth inhibition of L-CFU as well as CFU-GM and BFU-E was observed by rH-TNF at

concentrations of 1 to 100 U/mL. The inhibitory effect on L-CFU was significantly greater than that on CFU-GM. No correlation was observed between the inhibitory effect on L-CFU and the number of colonies formed in the cultures without rH-TNF. Preincubation of the progenitor cells in culture medium containing 20% fetal calf serum with up to 1,000 U/mL of rH-TNF for 24 hours did not result in the inhibition of colony growth of L-CFU or CFU-GM. The inhibitory effect of rH-TNF was neutralized by an anti-rH-TNF murine monoclonal antibody.

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TUMOR NECROSIS factor (TNF) was discovered by Carswell et al¹ in the sera of mice that had been primed with bacillus Calmette-Guerin and subsequently challenged with endotoxins and is known to be associated with in vivo and in vitro killing of tumor cells.¹⁻⁵ Although early studies indicated that TNF had no effect on nontransformed culture cells,^{4,5} some investigators reported that TNF showed cytotoxic activities against a certain subpopulation of mouse and human lymphocytes.^{6,7} All of these previous studies on TNF, however, were conducted with crude or partially purified TNF. Therefore, the possible presence of other cytokines such as interferon has remained a concern.⁸

Recently, the gene for human TNF has been cloned and expressed in *Escherichia coli*,^{9,10} and the product of this expression was isolated in a pure form and shown to produce necrosis of murine tumors in vivo. Using the recombinant TNF from different sources, we¹¹ and others¹²⁻¹⁴ found that recombinant human TNF (rH-TNF) suppressed the in vitro colony growth of human hematopoietic progenitor cells. In the present study we have investigated whether TNF shows a selective cytotoxicity on human hematologic malignancies and tested the effect of rH-TNF on the in vitro colony growth of human leukemia progenitor cells (L-CFU), granulocyte-macrophage progenitor cells (CFU-GM), and erythroid progenitor cells (BFU-E). We found that rH-TNF inhibited the colony growth of L-CFU, CFU-GM, and BFU-E in a dose-dependent manner and the inhibitory effect of rH-TNF was significantly greater on L-CFU than on CFU-GM at the concentration of 100 U/mL.

MATERIALS AND METHODS

Leukemia cells and bone marrow mononuclear cells. For the L-CFU colony assays, leukemia cells were obtained from 48 patients with acute myelogenous leukemia (AML). Bone marrow or peripheral blood containing more than 80% leukemia cells was drawn and anticoagulated with preservative-free heparin. For the CFU-GM assays, bone marrow was drawn from ten hematologically normal donors and 12 patients with AML, acute lymphoblastic leukemia (ALL), or non-Hodgkin's lymphoma (NHL) in complete remission (CR). For the BFU-E assays, bone marrow was obtained from 11 hematologically normal donors and three patients with NHL in CR. All procedures were performed with informed consent.

Assays for L-CFU and CFU-GM. To compare the effects of rH-TNF on L-CFU and CFU-GM, they were assayed under the same experimental conditions according to the method described by Minden et al¹⁵ with a slight modification. Leukemia cells or bone

marrow mononuclear cells were separated from RBC and mature granulocytes by Ficoll-Conray gradient centrifugation (400 g, 30 minutes) and depleted of T lymphocytes by a second Ficoll-Conray centrifugation after rosetting with 2-aminoethylthiuronium bromide-treated sheep RBC according to the method described by Madsen et al.¹⁶ The cells remaining in the interface were washed twice and plated in 0.5 mL of culture medium containing 0.3% agar (Difco Laboratories, Detroit), α -medium (GIBCO, Grand Island, NY), 20% fetal calf serum (FCS, GIBCO), 10% conditioned medium prepared with phytohemagglutinin-stimulated leukocytes as a source of colony-stimulating activity, and 1% diluted rH-TNF. Cultures containing 1×10^4 to 2×10^5 leukemia cells for the L-CFU assay or 1×10^5 bone marrow mononuclear cells for the CFU-GM assay were incubated for nine to 11 days at 37 °C in 5% CO₂ in air. All cultures were performed in triplicate. Each agar disk was transferred onto a slide glass, fixed, and stained with Harris' hematoxylin. Colonies (>40 cells) were counted under a microscope. In some experiments, cells were simultaneously tested that had been depleted of adherent cells before the T cell depletion by two cycles of adherence to plastic dishes pretreated with FCS overnight. In each experiment of L-CFU, some colonies were picked up with a fine Pasteur pipette and stained with May-Grünwald-Giemsa stain, and their blastic nature was confirmed by observing their morphology. In some experiments, cells at the same concentration as that for semisolid culture in α -medium with 20% FCS were preincubated with up to 1,000 U/mL of rH-TNF for 24 hours at 37 °C in 5% CO₂ in air, then washed three times, and subsequently assayed for L-CFU or CFU-GM. In another experiment, cells were used that had been frozen in a programmed freezer in the medium containing 10% dimethyl sulfoxide and 10% FCS and preserved at -196 °C.

Assays for BFU-E. One $\times 10^5$ unfractionated bone marrow mononuclear cells were plated in 0.5 mL of culture medium containing 0.3% agar, α -medium, 30% FCS, 1% bovine serum albumin (BSA, Sigma Chemical Co, St Louis), 2 U/mL erythropoietin (Connaught Laboratories, Willowdale, Ontario, Canada), and 1%

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diluted rH-TNF. All cells were freshly prepared. After incubation for 14 days, each culture was stained with 3,3'-diaminobenzidine tetrahydrochloride, and colonies consisting of 100 or more diaminobenzidine-positive cells were counted under a microscope.

rH-TNF. rH-TNF was kindly provided by Asahi Chemical Industry Co, Ltd, Tokyo. The rH-TNF was produced in *E coli* that expressed the gene encoding human TNF that had been identified in a human genomic DNA library¹⁷ by using a cloned cDNA encoding a portion of rabbit TNF as a probe.¹⁰ The TNF consisted of 155 amino acids with a molecular weight of 17,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It has over 99% purity and a specific activity of 2.2×10^6 U/mg protein. The unit was designated as the reciprocal of dilution that killed 50% of the TNF-sensitive L-M cells which were derived from clone 929 mouse L cells. rH-TNF was diluted with a diluent consisting of 5 mmol/L phosphate buffer (pH 7.4), 0.15 mol/L NaCl, and 0.1% gelatin and added to the culture medium shortly before plating. The dilution was prepared freshly before each experiment.

Neutralization of rH-TNF activity by anti-rH-TNF monoclonal antibody. One hundred microliters of rH-TNF (10^5 U/mL) was first incubated with 13 μ L of anti-rH-TNF murine monoclonal antibody (MoAb) (30 mg/mL, subclass IgG1, provided by Asahi Chemical Industry Co)¹⁸ for one hour at 37 °C. Controls included incubation of the MoAb without rH-TNF and incubation of purified normal mouse IgG (Zymed Laboratories, San Francisco) with rH-TNF. These mixtures were added to the culture medium just before plating.

Statistical analysis. Statistical analysis was done by Student's *t* test.

RESULTS

Effect of rH-TNF on the colony growth of L-CFU. L-CFU grew from the leukemia cells of 31 out of 48 patients studied with AML. Ten cases among them gave sufficient numbers of colonies (>30 colonies/plate) and were selected for further study. At concentrations of 1 to 100 U/mL of rH-TNF, a dose-dependent inhibition of L-CFU colony growth was found. The suppressive effect was statistically significant in nine out of ten cases at 100 U/mL of rH-TNF (Table 1). Although the extent of suppression varied among the cases tested, it showed no correlation with the number of

colonies of the control cultures grown without rH-TNF. At the concentrations of 1, 10, and 100 U/mL of rH-TNF, the mean percent inhibitions were $14\% \pm 9\%$ (mean $\pm$ SD), $50\% \pm 27\%$, and $72\% \pm 27\%$ respectively.

Effects of rH-TNF on the colony growth of CFU-GM. All of the ten hematologically normal bone marrows and 11 of 12 bone marrows from patients with AML, ALL, or NHL in CR gave sufficient numbers of colonies (>30 colonies/plate) for the study of CFU-GM. At concentrations of 1 to 100 U/mL of rH-TNF, a dose-dependent inhibition of CFU-GM colony growth was found. The suppressive effect was statistically significant in 18 of 21 cases at 100 U/mL of rH-TNF (Table 2 and 3). The colonies grown from bone marrow mononuclear cells of patients with AML, ALL, or NHL in CR were regarded as normal CFU-GM based on the morphological features of colony-composing cells with normal differentiation into neutrophils, eosinophils, or monocytes. At all the concentrations tested, the extent of inhibition on CFU-GM from patients with AML, ALL, or NHL in CR was similar to that on CFU-GM from hematologically normal donors. At the concentrations of 1, 10, and 100 U/mL of rH-TNF, the mean percent inhibitions were $15\% \pm 13\%$ (mean $\pm$ SD), $33\% \pm 11\%$, and $44\% \pm 12\%$ for the former groups and $15\% \pm 13\%$, $30\% \pm 11\%$, and $48\% \pm 7\%$ for the normal donors. At the concentration of 100 U/mL, the suppressive effect of rH-TNF on L-CFU was significantly greater than on CFU-GM from hematologically normal donors ($P = .018$) or CFU-GM from patients with AML, ALL, and NHL in CR ($P = .010$) (Fig 1). When the effect of rH-TNF on L-CFU or CFU-GM was tested after the depletion of adherent cells, a similar degree of suppression was observed, which suggests that TNF was as effective a suppressor either in the absence or presence of adherent cells (data not shown). For the leukemia cells or the bone marrow cells that had been first preincubated with up to 1,000 U/mL of rH-TNF for 24 hours, depleted of rH-TNF by washing and then plated for the assay, no significant suppression of L-CFU or CFU-GM was observed. Figure 2 illustrates a representative experiment among four tests; two

Table 1. Effect of rH-TNF on the Colony Growth of L-CFU

Case	Age	Sex	Cells	No. of Colonies Cultured With rH-TNF (U/mL)*			
				0	1	10	100
1	34	F	C†	38 $\pm$ 6	32 $\pm$ 5	24 $\pm$ 14	15 $\pm$ 5
2	40	F	C	2,020 $\pm$ 460	1,710 $\pm$ 720	500 $\pm$ 190	150 $\pm$ 160
3	36	F	C	43 $\pm$ 7	28 $\pm$ 2§	12 $\pm$ 8	6 $\pm$ 3
4	31	M	C	262 $\pm$ 65	226 $\pm$ 39	82 $\pm$ 25§	14 $\pm$ 3§
5	59	F	C	236 $\pm$ 14	183 $\pm$ 10	15 $\pm$ 4	<1
6	61	M	F‡	315 $\pm$ 14	283 $\pm$ 55	151 $\pm$ 35	32 $\pm$ 14
7	27	F	C	3,390 $\pm$ 250	3,260 $\pm$ 460	2,830 $\pm$ 280	2,520 $\pm$ 430§
8	76	M	F	1,010 $\pm$ 10	930 $\pm$ 110	650 $\pm$ 20	300 $\pm$ 90
9	67	M	C	129 $\pm$ 39	110 $\pm$ 8	83 $\pm$ 7	35 $\pm$ 5
10	26	M	C	687 $\pm$ 102	647 $\pm$ 137	617 $\pm$ 95	493 $\pm$ 32§
Mean percent inhibition $\pm$ SD				0	14 $\pm$ 9	50 $\pm$ 27	72 $\pm$ 27

*Mean number of colonies (>40 cells) $\pm$ SD per 1×10^5 cells of triplicate cultures.

†Cultured after cryopreservation.

‡Cultured fresh.

§ $P < .05$.

|| $P < .01$.

Table 2. Effect of rH-TNF on the Colony Growth of CFU-GM From Hematologically Normal Donors

Case	Age	Sex	Cells	No. of Colonies Cultured With rH-TNF (U/mL)*			
				0	1	10	100
1	40	M	F†	79 ± 18	76 ± 24	65 ± 16	43 ± 10§
2	24	F	F	131 ± 26	111 ± 8	87 ± 2	85 ± 21
3	27	M	F	172 ± 31	165 ± 17	127 ± 13	100 ± 29§
4	35	F	C‡	60 ± 11	40 ± 3	32 ± 9§	29 ± 4§
5	18	M	F	120 ± 35	101 ± 4	75 ± 18	69 ± 9
6	35	M	C	47 ± 10	29 ± 3	26 ± 9	25 ± 6§
7	32	M	F	177 ± 31	144 ± 14	137 ± 49	89 ± 8
8	35	M	F	157 ± 36	141 ± 14	112 ± 7	80 ± 12§
9	54	M	F	66 ± 14	66 ± 22	49 ± 12	28 ± 5§
10	69	M	F	118 ± 4	105 ± 23	99 ± 12	53 ± 7
Mean percent inhibition ± SD				0	15 ± 13	30 ± 11	48 ± 7

*Mean number of colonies (>40 cells) ± SD per 1×10^5 cells of triplicate cultures.

†Cultured fresh.

‡Cultured after cryopreservation.

§ $P < .05$.

|| $P < .01$.

of L-CFU and two of CFU-GM. Almost equal levels of L-CFU or CFU-GM suppression were observed for the fresh and the cryopreserved cells from the same donors (data not shown).

Effects of rH-TNF on the colony growth of BFU-E. Eight of 11 hematologically normal bone marrows and two of three bone marrows from patients with NHL in CR gave sufficient numbers of colonies (>30 colonies/plate). At concentrations of 1 to 100 U/mL of rH-TNF, a dose-dependent inhibition of the BFU-E colony growth was found. The suppressive effect was statistically significant in all ten cases at 100 U/mL of rH-TNF (Table 4). At the concentrations of 1, 10, and 100 U/mL of rH-TNF, the mean percent inhibitions were $23\% \pm 21\%$ (mean ± SD), $62\% \pm 24\%$, and $97\% \pm 4\%$ respectively.

Neutralization of the rH-TNF activity by anti-rH-TNF MoAb. The suppressive effect of rH-TNF on L-CFU or

CFU-GM at concentrations of 50 and 500 U/mL was neutralized by a murine MoAb specific to rH-TNF and not by normal mouse IgG, which indicates that the inhibition was caused by the rH-TNF and not by a contaminant. Neither normal mouse IgG alone nor the MoAb alone showed any effect on L-CFU or CFU-GM. Figure 3 illustrates a representative experiment among four tests; two of L-CFU and two of CFU-GM.

DISCUSSION

The present study shows that rH-TNF suppresses the in vitro colony growth of human L-CFU, CFU-GM, and BFU-E. Because the suppressive effect was neutralized by a murine MoAb specific to rH-TNF, the effect is considered to derive from rH-TNF itself. At the concentration of 100 U/mL, although the extent of inhibition on L-CFU varied considerably among patients (26% to 100%), the suppressive

Table 3. Effect of rH-TNF on the Colony Growth of CFU-GM From Patients With Leukemia or Lymphoma in CR

Case	Age	Sex	Diagnosis	Cells	No. of Colonies Cultured With rH-TNF (U/mL)*			
					0	1	10	100
1	48	F	AML (CR)	C†	67 ± 6	45 ± 6§	39 ± 7§	37 ± 7§
2	42	F	AML (CR)	C	103 ± 13	80 ± 2	54 ± 10§	48 ± 8§
3	33	M	AML (CR)	C	34 ± 3	38 ± 11	24 ± 2§	23 ± 3
4	34	M	AML (CR)	F‡	68 ± 5	62 ± 7	46 ± 6§	43 ± 10
5	37	M	AML (CR)	F	65 ± 21	60 ± 11	51 ± 11	43 ± 6
6	34	F	ALL (CR)	C	62 ± 7	49 ± 8	35 ± 2	29 ± 6§
7	31	F	ALL (CR)	F	52 ± 2	42 ± 13	34 ± 3§	39 ± 3§
8	46	M	NHL (CR)	C	98 ± 22	69 ± 7	55 ± 2	43 ± 13
9	31	F	NHL (CR)	F	307 ± 31	283 ± 67	213 ± 57	117 ± 18§
10	40	M	NHL (CR)	F	137 ± 16	123 ± 18	120 ± 3	80 ± 20
11	60	M	NHL (CR)	F	76 ± 10	61 ± 28	56 ± 7	39 ± 17
Mean percent inhibition ± SD					0	15 ± 13§	33 ± 11§	44 ± 12§

*Mean number of colonies (>40 cells) ± SD per 1×10^5 cells of triplicate cultures.

†Cultured after cryopreservation.

‡Cultured fresh.

§ $P < .01$.

|| $P < .05$.

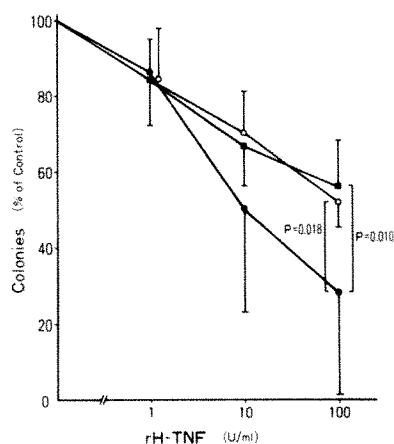


Fig 1. Comparison of the effects of rH-TNF on the colony growth of L-CFU and CFU-GM. The mean number of colonies from ten experiments on L-CFU from patients with AML (—●—), that from ten experiments on CFU-GM from hematologically normal donors (---○---), and that from 11 experiments on CFU-GM from patients with AML, ALL, and NHL in CR (—■—), which are expressed as a percentage of the number of colonies in each control culture without rH-TNF are shown. At the concentrations of 100 U/mL of rH-TNF, the inhibitory effect on L-CFU was significantly greater than that on CFU-GM from hematologically normal donors ($P = .018$) or CFU-GM from patients with AML, ALL, or NHL in CR ($P = .010$).

effect of rH-TNF on L-CFU was significantly greater than that on CFU-GM. No relationship was found between the extent of suppression of L-CFU and the number of colonies grown in the absence of rH-TNF. We did our best to perform these experiments under the same conditions for an accurate comparison of the effect of rH-TNF on L-CFU and CFU-GM. We defined L-CFU as colonies that had grown out of cells from bone marrow or peripheral blood containing more than 80% leukemia cells and the colony-composing cells that showed morphological characteristics of the original leukemia cells. It should be noted, however, that the morphological characteristics may not be enough for the evidence that the colony-composing cells in L-CFU were truly leukemic.

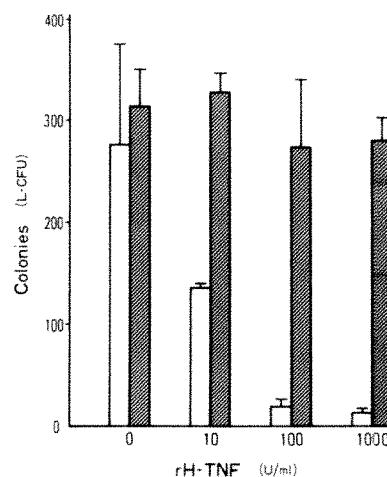


Fig 2. Effect of rH-TNF on the colony growth of L-CFU by preincubation study. No significant suppression was seen when 1×10^4 leukemia cells in 0.5 mL of α -medium with 20% FCS had been preincubated with rH-TNF for 24 hours at 37 °C in 5% CO₂ in air prior to the start of culture (hatched bars), whereas the suppressive effect of rH-TNF was evident when it was continuously added during the whole culture period (blank bars).

Therefore, we tested CFU-GM from patients with hematologic malignancies in CR under the same conditions. The results were almost the same as those on CFU-GM from normal donors, although, again, the morphological characteristics may not be enough for the evidence that the colony-composing cells in CFU-GM from patients with AML, ALL, or NHL in CR were truly normal.

Most previous studies using crude or partially purified TNF demonstrated that TNF did not affect nontransformed cells of mice or humans. However, our results indicate that TNF has inhibitory effects on normal human hematopoietic progenitor cells as well as leukemia progenitor cells. Degliantoni et al¹² independently found that rH-TNF from a different source suppressed the colony growth of CFU-GM while they were trying to prove that natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and

Table 4. Effect of rH-TNF on the Colony Growth of BFU-E

Case	Age	Sex	Cell Source	No. of Colonies Cultured With rH-TNF (U/mL)*			
				0	1	10	100
1	35	M	Normal	46 ± 19	11 ± 7†	0†	0†
2	53	F	Normal	146 ± 12	114 ± 9†	100 ± 14†	8 ± 3‡
3	69	M	Normal	68 ± 27	63 ± 26	15 ± 9†	0†
4	60	F	Normal	54 ± 13	44 ± 13	7 ± 4‡	0‡
5	62	F	Normal	52 ± 8	51 ± 14	29 ± 8†	<1‡
6	37	M	Normal	241 ± 11	207 ± 60	145 ± 7‡	32 ± 5‡
7	51	M	Normal	78 ± 22	72 ± 5	39 ± 3	2 ± 0‡
8	19	F	Normal	43 ± 15	36 ± 10	21 ± 4	0†
9	31	F	NHL (CR)	67 ± 27	29 ± 10	6 ± 2	0†
10	40	M	NHL (CR)	100 ± 20	86 ± 4	51 ± 1†	6 ± 4†
Mean percent inhibition ± S.D.				0	23 ± 21‡	62 ± 24‡	97 ± 4‡

All cells were prepared freshly.

*Mean number of colonies (>100 cells) ± SD per 1×10^6 cells of triplicate cultures.

† $P < .05$.

‡ $P < .01$.

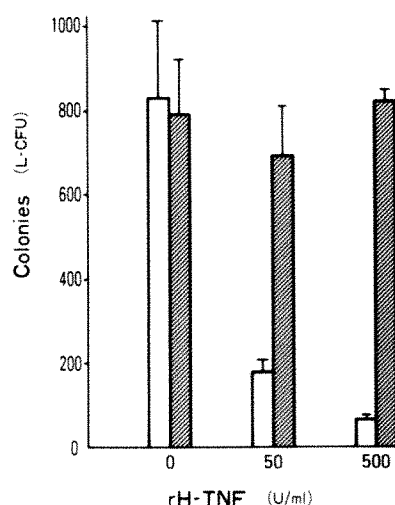


Fig 3. Neutralization of the rH-TNF activity by anti-rH-TNF MoAb. rH-TNF at 50 or 500 U/mL was incubated with anti-rH-TNF murine MoAb (hatched bars) or with normal mouse IgG (blank bars) for one hour at 37 °C and added to the L-CFU cultures just before plating.

NK cytotoxic factor are probably identical with TNF. Playfair et al⁶ reported that crude TNF showed cytotoxic activities against a certain subpopulation of mouse lymphocytes. Umeda et al⁷ observed a cytotoxic effect of TNF on lectin-activated human lymphocytes and the specific binding of TNF to the target cells, which indicated that normal lymphocytes also possessed TNF receptors on their cell surfaces. Recently, Beutler et al¹⁹ have reported the possible identity of TNF and the macrophage-secreted factor cachectin, which can bind to the receptors present on several normal murine tissues. They found, however, no detectable quantities of cachectin receptors on erythrocytes and lymphocytes, even though Sassa et al²⁰ from the same group had reported the inhibition of growth and the differentiation of transformed erythroid precursor cells (Friend's leukemia cells) by crude cachectin. On the other hand, some investigators have reported the ability of rH-TNF to stimulate the growth of several normal human cell lines.^{21,22}

After this paper had been submitted for publication, Broxmeyer et al²³ reported the suppressive effect of two types of human TNF (purified and recombinant) on bone marrow

hematopoietic progenitor cells from normal donors and patients with leukemia. They found that the suppression of leukemia cluster-forming cells by TNF was stronger than that of CFU-GM. These results appear to be consistent with ours. In the present study, the suppressive effect of rH-TNF on BFU-E appeared to be stronger than that on CFU-GM. However, we think, these results for BFU-E are unable to be simply compared with those for L-CFU or CFU-GM because these assays were done under different experimental conditions such as the FCS concentration,²² the incubation period, and the presence or absence of BSA.

The cloning of the gene for human TNF and subsequent recombinant technology have permitted the production of rH-TNF in large quantities for clinical use, and a phase I study has already been conducted in Japan. From our experience in the phase I study at our hospital, 100 U/mL of rH-TNF is a concentration that is easily attainable in patients. Besides, even 1 U/mL rH-TNF showed mild but definite suppressive effect on L-CFU, CFU-GM, and BFU-E in vitro. However, because the preincubation of the progenitor cells with up to 1,000 U/mL of rH-TNF for 24 hours did not result in the significant suppression of the colony growth of L-CFU and CFU-GM, the suppressive effect appeared to be cytostatic. In contrast, Broxmeyer et al²³ reported that 60 minutes of pulse exposure of normal bone marrow cells to TNF produced significant suppression of the colony growth of CFU-GM, BFU-E, and multipotential progenitor cells. Their results are not consistent with ours and suggest that the suppressive effect of TNF on hematopoietic progenitor cells is cytotoxic. This discrepancy is difficult to explain, although it may be due to differences in experimental conditions such as the presence or absence of FCS during pulse exposure time.

In conclusion, whether the suppressive effect is cytotoxic or cytostatic, rH-TNF has greater suppressive effect on L-CFU than that on CFU-GM in vitro. rH-TNF may be of value in the treatment of AML, although it may cause some hematologic toxicity because of the suppressive effect on normal hematopoietic progenitor cells.

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A New Variant of the α Subunit of Spectrin in Hereditary Elliptocytosis

By S. Lambert and S. Zail

A kindred is described in which two brothers with a poikilocytic variant of hereditary elliptocytosis (HE) were found to have a defect of spectrin dimer association and a decreased spectrin-band 3 ratio. Two-dimensional gel electrophoresis of limited tryptic digests of their spectrin revealed decreased amounts of the α I domain when compared with control digests and the appearance of two major peptides with mol wts of 43,000 and 42,000 and isoelectric points (5.75 to 5.85) more basic than the α I domain. Tryptic digests of spectrin from the asymptomatic mother of the two brothers were normal. Immunoblots of the two-dimensional gels using an antiserum to the α I

domain revealed that the 43,000- and 42,000-dalton peptides were derived from the α I domain, along with a series of lower mol wt peptides, some of which were below the detection limits of Coomassie blue-stained gels. Limit chymotryptic maps of ^{125} I-labeled tryptic peptides confirmed that the 43,000- and 42,000-dalton peptides were derived from the α I domain. This kindred represents a new structural variant of spectrin in HE in that the major abnormal tryptic peptides derived from the α I domain have lower mol wts and more basic isoelectric points than hitherto described.

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SEVERAL MOLECULAR abnormalities affecting the α I domain of spectrin have been described in mild hereditary elliptocytosis (HE), which is the most prevalent form of hereditary elliptocytosis, and in the closely related condition of hereditary pyropoikilocytosis (HPP).¹⁻³ The α I domain constitutes the N-terminal head region of the α chain, which together with the phosphorylated β I domain of the β chain is involved in spectrin tetramer and higher oligomer assembly.²⁻⁴ Molecular lesions of these domains lead to defective tetramer assembly and are thought to be causally related to the genesis of the elliptocytes and poikilocytes characteristic of HE and HPP.³

A feature of patients with mild HE or HPP who have structural defects of the α I domain is that limited tryptic digests of spectrin reveal a decrease or complete absence of this domain (80,000 daltons) on two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).^{2,3} In one structural variant, there is a reciprocal increase in a 74,000-dalton peptide, HE [Sp $\alpha^{1/74}$] and HPP [Sp $\alpha^{1/74}$],^{5,6} and in another there is an increase in several tryptic peptides of 46,000 daltons with isoelectric points between 5.25 and 5.35 and a peptide of 17,000 daltons with an isoelectric point of 5.4, HE [Sp $\alpha^{1/46}$] and HPP [Sp $\alpha^{1/46}$].^{5,7} (For the nomenclature of spectrin α chain defects, see ref. 3.) Two kindreds with HPP described by Knowles and co-workers⁸ and two kindreds with HE described by Marchesi and colleagues⁹ have increased amounts of tryptic peptides with mol wts of 50,000 and 21,000 and similar isoelectric points to the abnormal peptides of HPP [Sp $\alpha^{1/46}$]. A third structural variant in which the abnormal peptide has a mol wt of 65,000 has been described only in HE: HE [Sp $\alpha^{1/65}$].^{10,11}

In this study, we report a new variant of spectrin in a kindred with a poikilocytic variant of HE in which the α I domain is diminished on tryptic digestion, with the appearance of two major abnormal peptides with mol wts of 43,000 and 42,000, and in which the isoelectric points are considerably more basic (pI 5.75 to 5.85) than previously described. These peptides, together with several minor lower mol wt peptides, are shown to be derived from the α I domain, using immunoblots probed with an antibody to the α I domain. Limit chymotryptic maps of the 43,000- and 42,000-dalton peptides confirm that they are derived from the α I domain.

METHODS

Patients. The proband, M.S., aged 35 years, is a black South African miner who was initially investigated in 1982 after admission to hospital for abdominal pain. He improved with symptomatic therapy and has since had no recurrence of his symptoms. Both he and his younger brother (A.S.) (aged 30 years) were splenectomized in infancy, but details of these admissions are not available. Examination of the blood smears of both A.S. and M.S. revealed a predominance of elliptocytes (40% to 50%), with a considerable number of small, densely staining poikilocytes and some budding RBCs (Fig 1A). RBC indices for M.S. were: hemoglobin 15.0 g/dL; hematocrit 42%; MCHC 36 g/dL; MCV 69 fL; reticulocyte count 2.0%. RBC indices for A.S. were: hemoglobin 15.5 g/dL; hematocrit 43%; MCHC 36 g/dL; MCV 68 fL; reticulocyte count 4.0%. Autohemolysis for both patients varied between 30% and 40% and was partially corrected by glucose. Osmotic fragility of fresh RBCs was increased, with a median cell fragility of 0.48 g/dL NaCl in both patients (normal range 0.40 to 0.45 g/dL). Serum vitamin B₁₂ and RBC and serum folate were normal. Hematologic parameters in the mother, who was asymptomatic and has normal RBC morphology (Fig 1B) were within normal limits (hemoglobin 14.7 g/dL; MCV 89 fL; MCHC 34 g/dL; reticulocyte count 2.0%). The father is deceased. A.S. has a child who is unavailable for study. Incubation of venous blood of both M.S. and A.S. [within 6 hours of collection on ice in EDTA or acid-citrate-dextrose (ACD)] at 47 °C for 15 minutes leads to marked budding, distortion, and crenation of RBCs (Fig 1C). In contrast, no morphological alterations were seen in a normal control (Fig 1D) or in the mother (not shown).

Preparation of spectrin extracts. Blood was obtained from patients and controls after informed consent and approval of the

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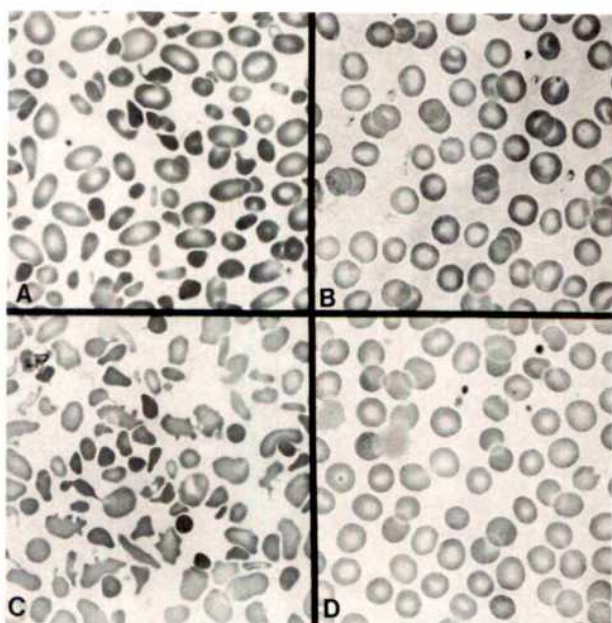


Fig 1. Photomicrograph of peripheral blood smears of (A) fresh RBCs of A.S.; (B) fresh RBCs of the mother of A.S.; (C) RBCs of A.S. heated for 15 minutes at 47°C; (D) RBCs of a normal control subject heated for 15 minutes at 47°C.

project by the Research Committee of the South African Institute for Medical Research. Venous blood was collected in ACD, kept on ice, and processed within 12 hours. Erythrocyte ghosts were prepared by hypotonic lysis of washed erythrocytes at 4°C in 30 vol of 10 mmol/L of Tris-HCl, 1 mmol/L of EDTA, 25 µg/mL of phenylmethylsulfonyl fluoride (PMSF) (dissolved at 25 mg/mL of isopropanol), pH 7.6, and centrifugation for 15 minutes at 30,000 g. The ghosts were washed four to five times in lysis buffer and then once in 0.25 mmol/L of EDTA, pH 8.0. Extracts of spectrin for tryptic digestion were obtained by incubating the ghosts (2 to 3 mg/mL) in 0.25 mmol/L of EDTA, pH 8.0, for 30 minutes at 37°C, and the supernate was collected after centrifugation at 150,000 g for 30 minutes at 4°C. Extracts of spectrin for determination of the proportion of spectrin dimers and tetramers in native membranes were obtained by incubating ghosts (2 to 3 mg/mL) in 0.25 mmol/L of EDTA, 25 µg/mL of PMSF, 2 µg/mL of pepstatin, 2 µg/mL of leupeptin, pH 8.0, for 24 hours at 0°C and centrifugation as before.

Tryptic digestion of spectrin. Spectrin (37°C) extracts were adjusted to a concentration of 40 mmol/L of Tris HCl, 20 mmol/L of Na acetate (pH 7.4) containing 1 mmol/L of dithiothreitol (DTT), and digested with 1:100 wt/wt L-(tosylamido 2-phenyl) ethylchloromethyl ketone-trypsin (231 U/mg, Worthington Biochemical, Freehold, NJ) for 18 hours at 0°C. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (BSA) as standard. Digestion was stopped by addition of 1 mmol/L of PMSF and snap-freezing in solid CO₂ and acetone. Samples were lyophilized and stored at -20°C.

Protein electrophoresis. Analytical SDS-PAGE on tube gels (4% acrylamide) was performed using the conditions of Fairbanks and colleagues.¹² One-dimensional gels stained with Coomassie blue were scanned with a recording densitometer (PU 8800, Pye Unicam, Cambridge, England). The areas under the spectrin and band 3 peaks were cut out and weighed and the spectrin-band 3 ratio was determined. Samples digested with trypsin were reconstituted in 9.5 mol/L of urea, 2% wt/vol NP-40, 5% mercaptoethanol, and 2%

ampholytes, pH 4.0 to 6.5 (Pharmalyte, Pharmacia Fine Chemicals, Uppsala, Sweden). Samples (150 to 200 µg of protein) were electro-focused for 6,500 V/h with a maximum of 400 V in 4% acrylamide gels containing 6.25% carrier ampholytes, pH 4.0 to 6.5, as described by O'Farrell.¹³ Equilibrium was reached under these conditions since electrofocusing for 10,000 V/h had no effect on the separation pattern. Second dimension SDS-PAGE was performed on 4% to 15% SDS acrylamide slab gels according to the method of Laemmli¹⁴ and fixed and stained with 0.1% Coomassie blue G-250 in 25% isopropanol, 10% acetic acid. Peptide mol wts were derived from logarithmic plots of mol wt against mobility, using as markers the mol wts assigned to erythrocyte membrane proteins by Lux.¹⁵ Preliminary experiments showed that these determinations correlated closely with mol wts derived using a commercial preparation of low-mol wt markers (Pharmacia). Electrophoretic transfer of fixed and stained gels was carried out according to the method of Jackson and Thompson.¹⁶ Gels were extensively destained in 10% acetic acid, washed in four changes of water, and incubated at 4°C in 0.025 mol/L Tris, 0.192 mol/L of glycine, and 10 g/L SDS for 2 hours. Proteins were then transferred using the method of Towbin and co-workers¹⁷ for 16 hours at 7.5 V/cm. Nitrocellulose blots were blocked with 3% gelatin at room temperature for 1 hour and treated essentially according to the method of Lawler and colleagues,¹¹ using an antiserum raised against the α I domain of normal spectrin (kindly supplied by Dr J. Lawler, St Elizabeth's Hospital, Boston). All wash buffers (except directly before color development) contained 0.05% Tween, and color development was performed using 3,3'-diaminobenzidine tetrahydrochloride as substrate. Blots were photographed using reflected light through a Wratten 47B gelatin filter to remove interference from Coomassie blue-stained peptides. Immunoblots of unfixed, unstained gels gave essentially similar blots but did not allow as direct a comparison of the immunoblot with the Coomassie blue-stained gel; therefore, immunoblots of fixed, stained gels were used preferentially.

¹²⁵I-peptide mapping. Coomassie blue-stained proteins were cut from the two-dimensional polyacrylamide gels and labeled with ¹²⁵I (557 mCi/mL, Amersham International, Amersham, England) by the technique of Elder and co-workers.¹⁸ The iodinated gel slices were digested with 50 µg of α -chymotrypsin (Worthington Biochemical) in 1 mL of 50 mmol/L of ammonium bicarbonate (pH 8.0) for 25 hours at 37°C. The supernates were lyophilized. Autoradiographs of two-dimensional separations on cellulose plates (Merck, Darmstadt, F.R.G.) were obtained as described by Speicher and co-workers.¹⁹

RESULTS

SDS-PAGE of isolated membranes. Spectrin-band 3 ratios in M.S. and A.S. were 0.70 and 0.74, respectively. The ratio in eight normal controls was 1.02 ± 0.06 . SDS-PAGE of isolated membranes of M.S. and a normal control subject is shown in Fig 2B and 2A, respectively. Membrane proteins other than spectrin appeared normal in M.S. and A.S.

Nondenaturing gel electrophoresis of 0°C spectrin extracts. The proportion of spectrin dimers in 0°C membrane extracts (as a percentage of the total dimer plus tetramer pool and excluding oligomeric forms of spectrin) was increased to 25% in M.S. and 30% in A.S. (Table 1). The percentage of dimers in the mother of the patients was 10%, which is at the upper limits of the range found in 10 normal control subjects.

Two-dimensional isoelectric focusing/SDS-PAGE of limited tryptic digests of spectrin. Limited tryptic digests of

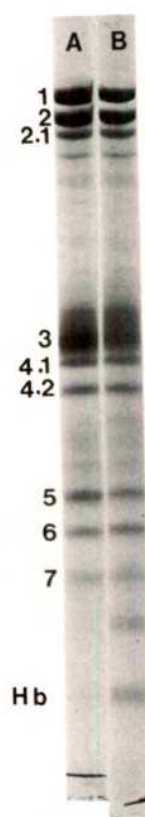


Fig 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of isolated RBC membranes of (A) a normal control subject; (B) patient M.S.

spectrin obtained from normal control subjects produced a pattern of peptides on two-dimensional SDS-PAGE similar to that described by others. A Coomassie blue-stained control digest (which was always run concurrently with patients' samples to identify minor variations in experimental conditions) is shown in Fig 3A, and the domains of spectrin are labeled according to the nomenclature of Speicher and co-workers.¹⁹ The position of the β IV peptide deserves comment. We found this peptide consistently in a position above the α I domain, in contrast to some workers^{2,8,9} who found it positioned below the α I domain. The β IV peptide has been found variably positioned by other workers,³ who, when using digestion conditions identical to that used in the present study, also found the peptide positioned above that of the α I domain.⁵

A structural polymorphism of the α II domain of spectrin, based on variations in mol wt and/or isoelectric point is present in American²⁰ and South African blacks and must be identified in both control and patient digests to distinguish any such variation from putative abnormal peptides in

patients' digests. The control digests in Fig 3A show a homozygous type 2 α II domain variant (3,000 dalton increase in mol wt, and more basic isoelectric point than does the common type I variant), and the representative peptides and their proteolytic products are ringed and joined by solid lines. Apart from the polymorphic variations in the α II domain, tryptic peptides maps identical to that shown in Fig 3A were obtained in more than 40 black control subjects. Figure 3C shows the corresponding limit tryptic digest of spectrin in the propositus, M.S. There is a marked reduction in the intensity of staining of the 80,000-mol wt α I domain (pI 5.35 to 5.45) and the generation of abnormal peptides with mol wts of 43,000 and 42,000 (large arrows in Fig 3C). These peptides have isoelectric points ranging between 5.75 to 5.85 that overlap the pI of the β IV domain. A faintly staining doublet of peptides with mol wts of 21,000 and 20,000 and a peptide with a mol wt of 16,000 are also shown at arrows in Fig 3C and represent abnormal peptides in M.S. In more than 40 black control subjects, the peptides with mol wts of 43,000, 42,000, 21,000, 20,000, and 16,000 were not observed, making it highly unlikely that the pattern observed in A.S. and M.S. represents a structural polymorphism in black subjects. The polymorphic α II-domain peptides and proteolytic products in M.S. show the features of a type 1/4 double heterozygote and are ringed in Fig 3C to distinguish them from the abnormal peptides.

An immunoblot of the control limited tryptic digest, probed with an antiserum to the α I domain, is shown in Fig 3B. An area corresponding to the α I domain and an overlapping peptide of 76,000 daltons stains most intensely, whereas a series of lower mol wt peptides of similar isoelectric point to the α I domain show less intense staining. These latter peptides are not visible on the corresponding Coomassie blue-stained gel in Fig 3A. A similar pattern of staining of the α I domain and lower mol wt peptides of similar isoelectric point are seen on the immunoblot of M.S. shown in Fig 3D. In addition, however, the abnormal peptides of mol wts of 43,000, 42,000, 21,000, 20,000, and 16,000 seen in the Coomassie blue-stained gel are also stained on the immunoblot (large arrows in Fig 3D) suggesting that these peptides are derived from the α I domain. Several other abnormal peptides also stained less intensely on the immunoblot of M.S. and are not visible on the Coomassie blue-stained gel. These are shown (small arrows) in Fig 3D. The immunoblot reveals that the peptides with mol wts of 21,000, 20,000, and 16,000 have multiple isoelectric points. In addition, one peptide shown (asterisk in Fig 3C) was not present in control digests and did not appear to be derived from the α I domain because it did not stain in the corresponding immunoblot. Coomassie blue-stained gels and immunoblots of A.S. were identical to those of M.S. Limited tryptic digests of the mother did not show any abnormality (not shown).

¹²⁵I-Peptide maps of the α I domain and the 43,000- and 42,000-dalton peptides. Further evidence that the major abnormal peptides of 43,000 and 42,000 daltons were fragments of the α I domain of spectrin in patient M.S. was shown by high-resolution ¹²⁵I-peptide maps of these tryptic peptides. Limit chymotryptic ¹²⁵I-peptides of the 43,000- and 42,000-dalton peptides (Fig. 4C and D) are contained within the

Table 1. Proportion of Spectrin Dimers in 0 °C Membrane Extracts

Subjects	Spectrin Dimers (%)	
	Dimers	Dimers + Tetramers × 100
M.S.	25	
A.S.	30	
Mother	10	
Controls (range) (n = 10)	5-10	

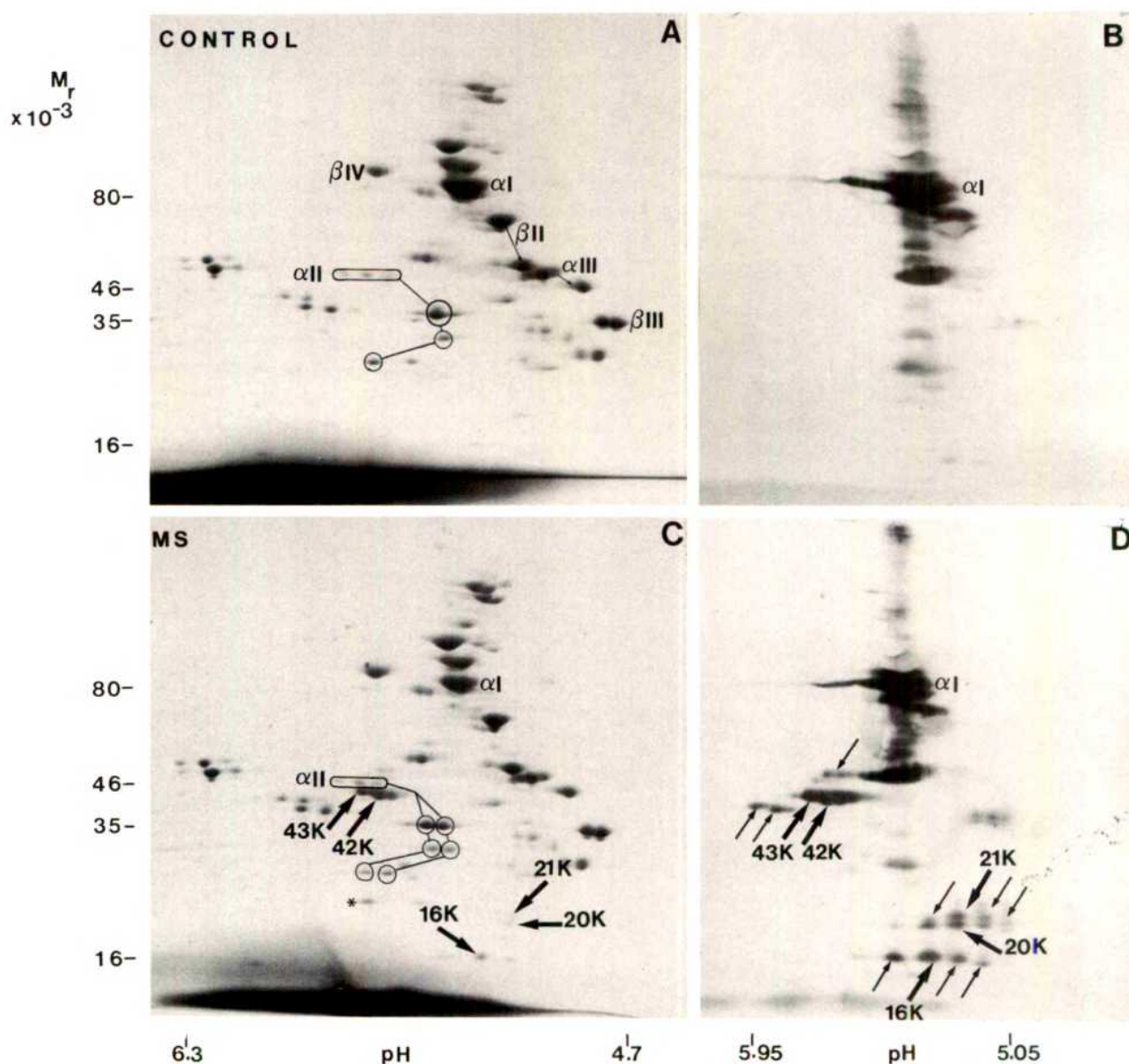


Fig 3. Two-dimensional isoelectric focusing/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of tryptic digests of spectrin extracts of a normal control subject (A and B) and patient M.S. (C and D). Coomassie blue-stained and fixed gels are shown on the left (A and C); the immunoblots (B and D) of the stained and fixed gels are shown on the right. An antiserum against the α I domain was used to probe the blots. The position of some of the tryptic domains of spectrin are indicated. Large arrows in C and D show the abnormal peptides generated in digests of M.S. present in both the Coomassie blue-stained gels and the immunoblot. The small arrows in D depict minor peptides derived from the α I domain but are visible only on the immunoblot. The peptide (asterisk; C) is not present in control digests and does not stain on the corresponding immunoblot. The ringed peptides in A and C represent the polymorphic α I domain and its proteolytic products.

map of ^{125} I-peptides of the remnant α I domain of spectrin of M.S. (Fig 4B). Two ^{125} I-peptides (arrows) in this map of the α I domain of M.S. (Fig 4B) showed altered mobility when compared with the map of the control α I domain (Fig 4A). These changes were not consistent in different limit chymotryptic maps of the α I domain of M.S., A.S., and other control subjects, however, and are therefore not regarded as being of significance.

DISCUSSION

The affected members of this kindred show a significant degree of elliptocytosis, with considerable numbers of dense,

bizarre poikilocytes (Fig 1A). They also have a mild defect of spectrin self-association, as manifested by an increase in the proportion of spectrin dimers in 0 °C low ionic strength extracts of isolated membranes. We believe that these patients should probably be classified as a poikilocytic variant of HE,⁹ rather than HPP, since the degree of elliptocytosis is in excess of that seen in HPP. Moreover, the altered heat stability is more consistent with HE, since the RBCs of these patients do not show gross fragmentation when heated to 47 °C. The distinction from HPP is, however, more difficult in this kindred because both patients show a decrease in the spectrin-band 3 ratio, which is a characteris-

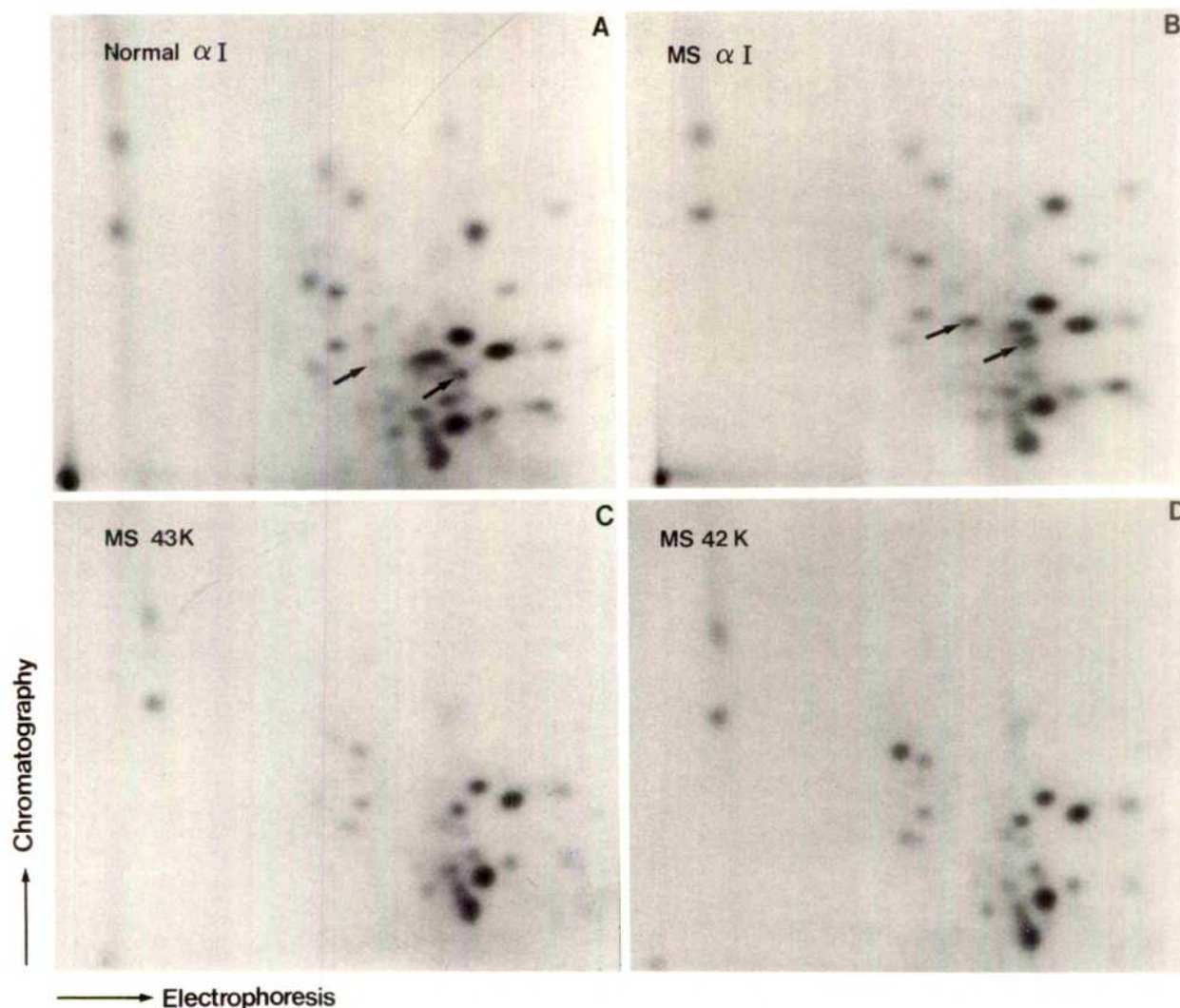


Fig 4. Limit chymotryptic ^{125}I -peptide maps of tryptic peptides of spectrin: (A) αI domain of normal control; (B) αI domain of M.S. (C and D) 43,000- and 42,000-dalton peptides of M.S. The arrows depict peptides in the maps of the αI domains of both the control subject and M.S. that exhibit changes in mobility not consistent between different experiments and therefore not regarded as being of significance.

tic feature of HPP.²¹ The mode of transmission is unfortunately obscure, since no data are available for the father, and the mother appears clinically, functionally, and biochemically normal.

The structural abnormality of the αI domain of spectrin in patients M.S. and A.S. is associated with defective tetramer formation, as manifested by an increased proportion of dimers in native membranes, and is presumably the cause of the clinical abnormality. Defects of the αI domain of spectrin in HE and HPP manifest on limited tryptic digests of spectrin as a decrease or absence of the αI domain. This presumably reflects a conformational change in the αI domain that renders it more susceptible to proteolysis. Some kindreds with mild HE or HPP demonstrate reciprocal generation of increased amounts of a 74,000-dalton peptide,^{5,6} whereas others show increased amounts of lower mol wt peptides variously estimated at between 46,000 and 50,000 daltons and 17,000 and 21,000 daltons.^{5,7-9} In the latter group, Lawler and co-workers^{5,7} found that the 46,000-dalton peptides had isoelectric points (5.25 to 5.35) that were

acidic relative to the αI domain (pI 5.3 to 5.4), whereas the 17,000-dalton peptides focused at approximately the same pI as the αI domain. The 50,000- and 21,000-dalton peptides described by Knowles and colleagues⁸ and Marchesi and co-workers⁹ in HPP and HE, respectively, had a similar orientation. A third variant, HE[Sp $\alpha^{1/65}$] had increased amounts of a peptide of mol wt 65,000.^{9,10} In our studies, the αI domain of normal spectrin focuses at a pI of 5.35 to 5.45, which is close to that found by Lawler and co-workers,^{5,7} but the abnormal 43,000- and 42,000-dalton peptides in M.S. and A.S. have isoelectric points (5.75 to 5.85) that are clearly basic relative to the αI domain. Because we also show that the 43,000- and 42,000-dalton peptides are derived from the αI domain on the basis of their cross-reactivity with an antiserum to the αI domain (Fig 3) and their sharing of common peptides on high-resolution ^{125}I -peptide mapping (Fig 4), patients M.S. and A.S. constitute a new variant of the αI domain in HE. Other changes are also apparent in our patients. The minor abnormal peptides at 21,000, 20,000, and 16,000 focus on Coomassie blue-stained gels and immu-

noblots (Fig 3D) at isoelectric points acidic to the α I domain, with the exception of one 16,000-dalton peptide on the immunoblot that has an isoelectric point similar to that of the α I domain. The immunoblot of MS (Fig 3D) also shows fainter staining peptides (small arrows) above and below the abnormal 43,000- and 42,000-dalton peptides that may be precursors and products, respectively, of the major abnormal peptides.

While this work was in progress, two preliminary reports by Marchesi and co-workers^{22,23} appeared, describing two black families with HE in whom abnormal tryptic peptides of

50,000 daltons, derived from the α I domain but with isoelectric points more basic than the α I domain, have been found. The variant spectrin described in the present study appears to be different from that described by Marchesi and co-workers²³ in that the major abnormal tryptic peptides have a considerably lower mol wt.

ACKNOWLEDGMENT

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Platelet Antithrombin Defect in Malignancy: Platelet Protein Alterations

By Elaine M. Sloand, Dianne M. Kenney, Francis C. Chao, Jack Lawler, and James L. Tullis

Sixty-eight patients with malignant disease were divided into two groups based on the results of the platelet antithrombin test (PAT). The normal group had a PAT clotting time ranging from 21.4 to 29.8 seconds, which was equivalent to 25% to 65% inactivation of the 2 U of thrombin added to the test system. The other group showed abnormal PAT clotting time, <21.4 seconds or <25% thrombin inactivation. The polypeptide composition of platelets from the two patient groups was analyzed by sodium dodecyl sulfate (SDS)-electrophoresis on 7.5% polyacrylamide gels. A polypeptide of 180,000 apparent mol wt was decreased or absent in both Coomassie blue- and Alcian blue-stained gels of the platelets from patients whose PAT was abnormal; this polypeptide comigrated with purified platelet thrombospondin. Tritium labeling of platelet surface glycoproteins by the periodate-borohydride method followed by two-dimensional electrophoresis was performed on platelets of seven patients with abnormal

PAT. When they were compared with ten patients with normal PAT, a glycoprotein of 140,000 apparent mol wt with a pI of 4.5 to 5.2 was decreased in platelets of all seven patients with abnormal PAT. Nitrocellulose replicas of one-dimensional gels of platelets from 13 of 14 patients with abnormal PAT showed decreased reaction with an anti-human platelet glycoprotein antiserum. Platelets of these same patients also showed a decreased or absent platelet agglutination induced by ristocetin. Patients with normal PAT had a mean agglutination slope of 1.25 ± 0.6 ($n = 26$) as compared with 0.37 ± 0.34 ($n = 26$) for the abnormal PAT group ($P < .001$). Results indicate that platelets from a subpopulation of tumor patients characterized by decreased platelet antithrombin activity have alterations in two platelet glycoproteins, identified as GPIb and thrombospondin.

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THE CLOTTING ACTIVITY of α -thrombin can be neutralized by intact human platelets. This activity is referred to as platelet antithrombin.¹ Thrombin inactivation by normal platelets has been shown to be membrane dependent for two-thirds of the activity and secondary to the release of intracellular platelet fibrinogen for the other third.² A simple two-stage assay has established the normal physiologic range³ and has been used to demonstrate a decrease in platelet antithrombin activity in certain pathologic states. This includes patients with primary (congenital) deficiencies⁴ as well as patients with apparent acquired deficiency secondary to malignant states.⁵ In a separate clinical study, one third of the patients with advanced/disseminated adenocarcinomas, melanomas, and myeloproliferative disorders were found to have a consistent decrease in platelet antithrombin activity.⁶

The present study was designed to determine whether changes in platelet proteins could be correlated with abnormal platelet antithrombin activity. A group of patients with malignant disease and reduction in platelet antithrombin activity was compared with a group of patients with similar malignancies in whom thrombin inactivation by platelets was normal. The platelet proteins from these two groups of patients were analyzed by several electrophoretic and immunochemical techniques. The results demonstrate that two platelet proteins are altered in cancer patients whose platelets show defective inactivation of thrombin. One shows the characteristics of the membrane glycoprotein Ib (GPIb) and the other shows an α -granule component, thrombospondin.

MATERIALS AND METHODS

Patient population. Sixty-eight patients from the medical oncology ward of the New England Deaconess Hospital were divided into two groups on the basis of platelet antithrombin test (PAT) results. All were part of a larger clinical study involving platelet antithrombin determinations on >200 patients with advanced malignant disease.⁶ Malignancies studied included ovary, lung, colon, melanoma, and lymphoma. Patients who were receiving aspirin, nonsteroidal antiinflammatory agents, or vinca alkaloids were excluded. As established previously,⁴ results of PAT measurement

follow a bimodal distribution. Patients with abnormal PAT had a clotting time <20.5 seconds (PAT measurement is also described in the Results section). Fourteen of the 37 patients with an abnormal PAT and 15 of the 31 patients with a normal PAT were not on current chemotherapy. The members of each group who were receiving chemotherapy had equal exposure to standard agents such as fluorouridine (FUDR), cytoxan, cisplatin, 5-fluorouracil (5-FU), and adriamycin.

Platelet preparation. Venous blood was drawn from the 68 patients and from three normal individuals (healthy controls) and mixed with acid-citrate-dextrose (ACD) [National Institutes of Health (NIH), Formula A] and EDTA anticoagulant solution to yield final concentrations of 15% (vol) ACD and 5 mmol/L of EDTA. After centrifugation at room temperature at 130 g for 15 minutes, the platelet-rich plasma (PRP) was removed, a 20% vol of ACD-EDTA was added, and the platelets were pelleted by centrifugation at 1,200 g for 15 minutes. Platelets were washed twice by resuspension in Tris-buffered saline (TBS) containing 10% ACD-EDTA. Platelet aliquots used for the PAT were resuspended without anticoagulants and adjusted at 2×10^9 /mL. For electrophoresis, platelets were resuspended at 1×10^9 /mL in 67 mmol/L of Tris-HCl buffered 100 mmol/L of NaCl with 5 mmol/L of EDTA and 5 mmol/L of iodoacetamide.

Assay for platelet antithrombin activity. Platelet antithrombin activity was measured by a two-stage assay described previously.^{3,4} Washed platelets (0.5 mL) were incubated for 10 minutes at room

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temperature with 0.5 mL of thrombin solution (Parke-Davis, Morris Plains, NJ) at 4 NIH U/mL. Commercial thrombin gave results comparable to those of purified human thrombin.² After incubation, 0.2 mL of the reaction mixture was added to 0.2 mL of fibrinogen (2 mg/mL) prewarmed at 37 °C for 5 minutes. The clotting time was then determined. The variation between duplicate samples averaged 0.7 seconds (range 0 to 1.5 seconds).

SDS-Polyacrylamide gel electrophoresis (PAGE). Polypeptides were fractionated by SDS-PAGE according to the procedure of Laemmli⁷ using a 7.5% polyacrylamide resolving gel and a 4% stacking gel. Washed platelets were solubilized with 3% SDS and boiled for 3 minutes and were then treated with 10 mmol/L of dithiothreitol for 1 minute at 100 °C. Equivalent protein concentrations (20 to 80 µg) were applied in each analysis of platelets from patients with normal and abnormal PAT.

Gels were stained with Coomassie blue and Alcian blue. For Alcian blue staining,⁸ polyacrylamide gels were fixed in 12.5% trichloroacetic acid for 30 minutes and were then treated for 50 minutes at room temperature with 7% periodic acid in 3% acetic acid. The fixed gels were washed twice with 200 mL of distilled water and soaked in 0.5% metabisulfite for 30 minutes. The gels were washed and stained with 1% Alcian blue (in 3% acetic acid) overnight and destained in 7% acetic acid. Standard mol wt marker proteins were: spectrin heterodimer (240,000 and 220,000) – galactosidase (130,000), phosphorylase A (94,000), albumin (68,000), glutamate dehydrogenase (56,000), creatinine kinase (40,000), and carbonic anhydrase (29,000).

Radiolabeling of surface carbohydrate moieties. Surface sialic acid residues of glycoproteins were labeled with ³H by the method of Steiner and colleagues⁹ with some modifications. Platelets washed twice in Tyrodes' solution containing 5 mmol/L of EDTA, buffered at pH 6.8 with 10 mmol/L of *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) (HTE), were resuspended in a 1- to 2-mL vol of HTE at 0.5×10^9 cells/mL. Sodium periodate was added to a final concentration of 2 mmol/L, and the reaction mixture was incubated at 4 °C in darkness for 20 minutes. Unreacted periodate was removed by two washes with HTE at pH 7.6, and the platelets were resuspended in 1 to 2 mL of the same buffer. [³H]NaBH₄ (5 mCi in 50 µL of cold 0.01 mol/L of NaOH) was added to a final concentration of 500 µCi/ 0.5×10^9 platelets and incubated for 30 minutes at room temperature with periodic agitation. Radiolabeled platelets were washed twice, resuspended in HTE (pH 6.8), and extracted with 0.5% NP-40 in the presence of 5 mmol/L of iodoacetamide. Extracts of radiolabeled platelets, routinely frozen at -20 °C, were thawed and clarified by centrifugation at 1,200 g for 15 minutes and subjected to one- or two-dimensional SDS-PAGE. Polyacrylamide gels containing ³H-labeled proteins were prepared for fluorography by being impregnated in 22% (wt/vol) diphenyloxazole in dimethylsulfoxide (DMSO) as described by Bonner and Laskey.¹⁰ This technique was also used to analyze xylocaine-treated platelets (described later).

Two-dimensional gel electrophoresis. Two-dimensional isoelectric focusing-SDS-gel electrophoresis was performed by a method modified from O'Farrell.¹¹ Extracts of radiolabeled platelets were prepared for isoelectric focusing by adding solid urea and dithiothreitol to final concentrations of 9.5 mol/L and 100 mmol/L, respectively. Samples containing 20 µg of protein were applied to the isoelectric focusing gels, which were composed of 3.5% acrylamide, 3.2% Triton X-100 (Fisher Scientific, Springfield, NJ), 4% Ampholines (pH range 5 to 8), 0.4% Ampholines (pH range 4 to 6), 0.6% Ampholines (pH range 3.5 to 10), and focused for 16 hours at 400 V and for 2 hours at 1,000 V. The pH values (reflecting relative isoelectric points of separated proteins) were measured from Ampholines eluted from 5-mm slices of duplicate gels that had been focused in parallel without protein samples. Extraction with NP-40

allowed determination of the relative isoelectric points of nondenatured polypeptides by two-dimensional electrophoresis and of mol wt by SDS-PAGE on polypeptides on the same sample.

Isolation of glyocalicin and thrombospondin. Glyocalicin was isolated from platelet concentrates that were unsuitable for clinical transfusion, using the methods of Okumura and co-workers¹² and Clemetson and colleagues,¹³ with some modification. The platelets were washed three times with 0.02 mol/L of Tris-HCl (pH 7.6), 0.15 mol/L of NaCl and 1 mmol/L EDTA (TSE) solution, resuspended in 1.0 mol/L of NaCl, 0.02 mol/L of Tris-HCl (pH 7.6) and 1 mmol/L of EDTA, incubated while being stirred at 22 °C for 1 hour, and then centrifuged at 50,000 g for 1 hour at 4 °C. The supernatant was dialyzed at 4 °C against 0.02 mol/L of Tris-HCl (pH 7.6) containing 1 mmol/L of EDTA to reduce the NaCl concentration to 0.15 mol/L and applied to a column (0.9 × 9 cm) of wheat germ agglutinin (WGA) coupled to Sepharose 6-B (Pharmacia, Sweden). Stepwise elution was performed with TSE alone and with TSE containing 4.5 mmol/L of *N*-acetylglucosamine (GlcNAc) or 450 mmol/L of GlcNAc. The 450-mmol/L GlcNAc eluate was dialyzed against 6 mmol/L of potassium phosphate buffer (pH 6.8) with 0.2 mmol/L of EDTA for six changes over 72 hours and then applied to a column (2.5 × 8 cm) of freshly prepared hydroxylapatite. Stepwise elution was performed with 6, 40, and 200 mmol/L of potassium phosphate buffer containing 0.2 mmol/L of EDTA. The fraction eluted with 40 mmol/L of potassium phosphate showed a single band of 130,000 apparent mol wt when analyzed by SDS-PAGE followed by Alcian blue staining. Purified glyocalicin was stored at -20 °C. Thrombospondin was isolated from the supernatants of washed platelets that had been treated with thrombin by the method described previously.¹⁴

Antiserum preparation. Antiplatelet glyocalicin antiserum (raised in rabbits) was obtained from PRP, Inc. (Newton, MA). Antithrombospondin antiserum was prepared in mice. Female BALB/c mice, aged 6 to 10 weeks, were immunized by intraperitoneal (IP) injections with 0.1 mg of purified human platelet thrombospondin and emulsified in complete Freund's adjuvant followed by two booster IP injections (at 3-week intervals) of 50 µg of antigen in TBS/2 mmol/L of CaCl₂ in incomplete Freund's adjuvant. Serum containing antibody was prepared from blood collected 3 weeks after the second booster injection.

Electroblotting and immune staining. Washed platelets were solubilized with SDS and fractionated by SDS-PAGE on 7.5% slab gels.⁷ Fractionated polypeptides were electrophoretically transferred to nitrocellulose paper at 30 V for 20 to 24 hours at 10 °C in pH 8.3 buffer containing 25 mmol/L of Tris, and 192 mmol/L of glycine as described,¹⁵ except that no methanol was used. Nitrocellulose replicas were washed in 0.15 mol/L of NaCl, incubated with phosphate-buffered saline (PBS; pH 7.5) containing 5% bovine serum albumin (PBS-albumin) for 1 hour at 22 °C, then washed with five changes of 0.15 mol/L of NaCl solution over 30 minutes. The protein blots were incubated with either the monospecific antisera against human platelet glyocalicin or thrombospondin, washed, then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for glyocalicin), or goat anti-mouse IgG (for thrombospondin) (10 µL/10 mL TBS, Cooper Biomedical [Cappel Division], Malvern, PA) for 1 hour at 22 °C. Bound anti-IgGs were detected with 4-chloro-1-naphthol (0.6 mg/mL) and 0.3% hydrogen peroxide in a solution of 20% methanol and 50 mmol/L of Tris-HCl (pH 7.6).

Measurement of ristocetin-induced agglutination. Formalin-fixed platelets were prepared for ristocetin agglutination by a method modified from Coller and co-workers¹⁶ using Gaintner's buffer.¹⁷ A single batch of pooled normal citrate anticoagulated plasma (diluted 1:2) was used as the standard source of factor VIII von Willebrand factor (vWf). The platelet suspension (0.35 mL) was incubated with 0.1 mL of the plasma at 37 °C, and aggregation

was induced by the addition of ristocetin (50 μ L of 1.5 mg/mL). The steepest slope of the agglutination curve was used to assess ristocetin agglutination as described by Weiss.¹⁸

Xylocaine-treatment of platelets. Washed platelets in TBS (1×10^9 /mL) were incubated with xylocaine (40 mmol/L) at 37 °C for 3 hours as described by Collier.¹⁹ Control samples were incubated in the presence of 5 mmol/L of EDTA without xylocaine. After incubation, platelets were washed twice in Tris-buffered saline and prepared for PAT measurement and surface labeling as described previously.

Other methods. Platelet sizing and WBC determinations were done electronically using a Coulter Counter (Model-S). All platelet preparations were 99.9% leukocyte-free. Protein determinations were performed by the method of Lowry and colleagues.²⁰ Individual polypeptides in electrophoresis gels and autoradiograms were estimated by densitometric scanning using a 580-nm filter on a Hoeffer gel densitometer.

RESULTS

The platelets from 68 tumor patients were studied. Of this group, 37 had abnormal platelet antithrombin. The PATs in the abnormal group ranged from 17.0 to 20.5 seconds with an average of 18.6 seconds (SD 0.96). The PATs of the normal group ranged from 21.4 to 29.8 seconds with an average of 25.4 seconds (SD 1.97). The coefficient of variation was 5.2% for the abnormal group and 7.8% for the normal group.

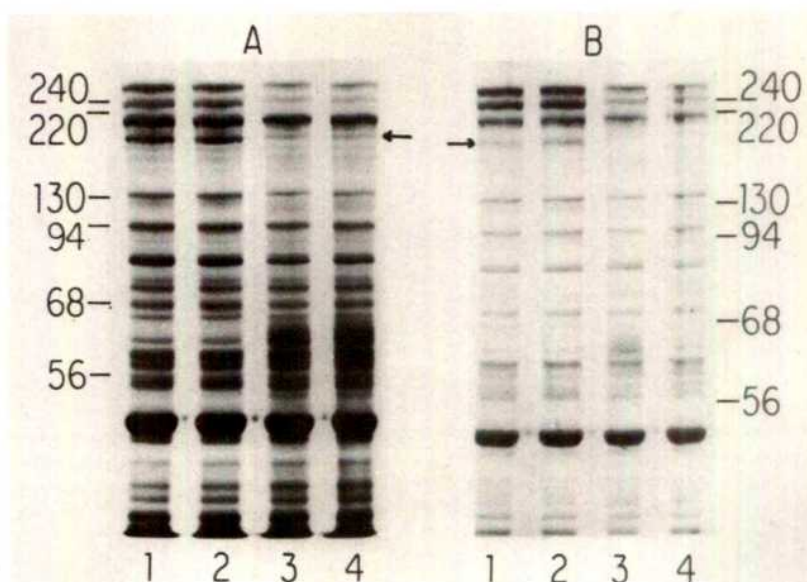
When the polypeptide composition of platelets from the 37 patients with abnormal PAT was analyzed by SDS-PAGE and compared with the protein composition of platelets from 31 patients with normal PAT and three healthy individuals, a marked reduction in a polypeptide of 180,000 apparent mol wt was demonstrated; this difference was evident when gels were stained with either Alcian blue or Coomassie blue (Fig 1A and B). Staining by Alcian blue was found to be more sensitive than that by Coomassie blue, however, in demonstrating alterations of 180,000 mol wt polypeptide. This polypeptide could be readily detected by Alcian blue stain when only 20 to 40 μ g protein from normal platelets was

applied to the gels, whereas 40 to 80 μ g of normal platelet protein was required to detect this polypeptide by Coomassie blue staining. By means of Alcian blue staining, the 180,000-mol wt polypeptide was shown to be decreased or absent in 36 of 37 samples with abnormal PAT and present in the 34 control samples with normal PAT. Purified human platelet thrombospondin, analyzed in parallel with platelet samples, exhibited identical electrophoretic mobility and staining characteristics (under reducing conditions) to this 180,000-mol wt polypeptide (Fig 2). Furthermore, the nitrocellulose replicas of SDS gels of normal platelet proteins showed reaction of this 180,000-mol wt polypeptide with a monospecific antiserum against human platelet thrombospondin; this polypeptide also was decreased or absent in platelets from patients with abnormal PAT (Fig 2). On this basis, the decreased polypeptide in platelets from patients with abnormal PAT was identified as thrombospondin.

Platelet surface glycoproteins from seven of the cancer patients whose platelets showed an abnormal PAT and those from ten individuals with a normal PAT were radiolabeled with ³H and analyzed by two-dimensional isoelectric focusing/SDS-electrophoresis and autoradiography (Fig 3). Under the precise experimental conditions described previously in the Materials and Methods section, platelets from patients with normal PAT showed three major radiolabeled polypeptides. These polypeptides had apparent reduced mol wts of 140,000, 120,000, and 100,000, and relative pIs of 4.5 to 5.2, 5.3 to 5.5, and 5.3 to 5.5, respectively. The polypeptide of ~140,000 mol wt and pI 4.5 to 5.2 was diminished or absent in all seven patients with low PAT. Quantitation of labeled polypeptides by densitometric scanning of autoradiograms showed that the ratio of the densities of the 140,000- to the 120,000-mol wt polypeptide was significantly lower in the abnormal group than in the normal patient group, whereas the densities of the 120,000-mol wt polypeptide were similar in both groups.

Studies were undertaken to examine platelets of the two patient groups with a specific immunochemical probe for

Fig 1. Polypeptide composition of platelets from individuals with normal and abnormal platelet antithrombin test (PAT). Washed platelets were solubilized in 3% sodium dodecyl sulfate (SDS) and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels under reducing conditions. Gels were stained with Coomassie blue (A) and Alcian blue (B): lane 1, healthy control; lane 2, patient with normal PAT; lanes 3 and 4, patients with abnormal PAT. A polypeptide at 170,000 apparent mol wt is diminished in platelets from patients with abnormal PAT (arrows). For the Alcian blue-stained gels, the tracking dye was run off the leading edge for 30 minutes to improve resolution of high mol wt components. Positions of standard mol wt proteins are as indicated.



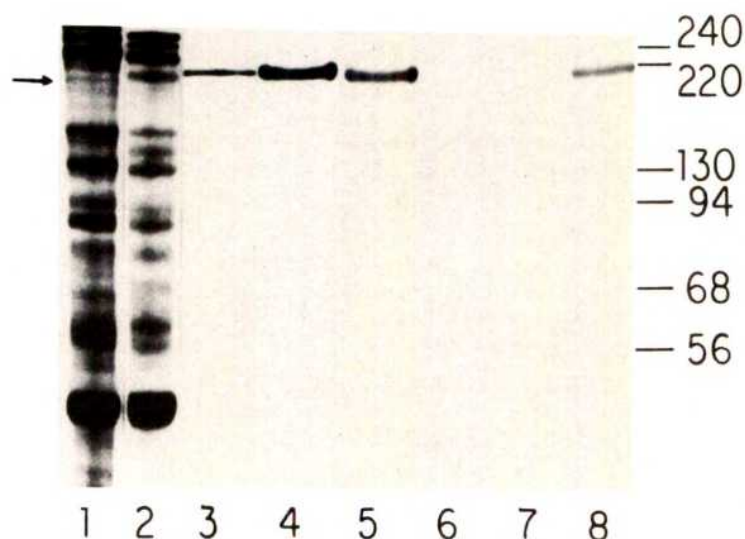


Fig 2. Decreased platelet thrombospondin in patients with low platelet antithrombin test (PAT). Washed platelets and purified thrombospondin were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and stained with Coomassie blue (lanes 1 through 3). Lane 1, platelets from a patient with abnormal PAT; lane 2, platelets from a patient with normal PAT; lane 3, purified thrombospondin. Nitrocellulose replicas of polyacrylamide gels containing purified thrombospondin (lane 4) and platelets from a healthy control (lane 5), a patient with normal PAT (lane 6), and two patients with abnormal PAT (lanes 6 and 7) were stained by horseradish peroxidase (HRP)-conjugated anti-mouse IgG after incubation with an antiserum monospecific for human platelet thrombospondin. The 180,000-mol wt polypeptide is reduced in lane 1 (arrow) and is absent in lanes 6 and 7. Position of standard mol wt proteins are shown on right.

GPIb—a polyclonal antiserum against purified human platelet glycoprotein. The antiserum (raised in rabbits) reacted with highly purified glycoprotein (mol wt 130,000) and a single, ^3H -labeled polypeptide of apparent reduced mol wt 140,000 when evaluated by staining of nitrocellulose replicas

of SDS gels containing isolated glycoprotein and the fractionated polypeptides of ^3H -labeled, normal platelets (Fig 4).

Platelet GPIb from 14 tumor patients with abnormal PAT and seven normal control subjects were analyzed by the immune replica technique, using the monospecific antiglycoprotein antiserum. There was a reduction in the intensity of staining of the polypeptide corresponding to GPIb in 13 of the 14 patients with abnormal PAT when compared with

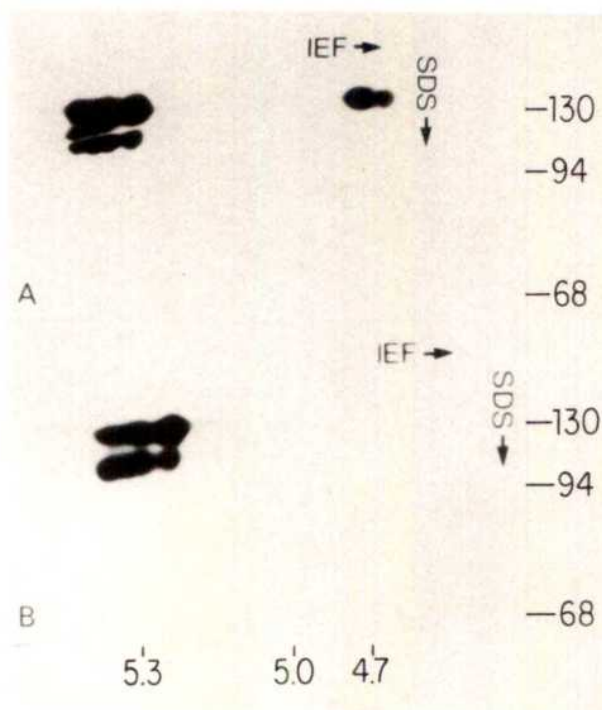


Fig 3. ^3H -labeled glycoproteins of platelets from a patient with abnormal platelet antithrombin test (PAT). Platelets from patients with normal (A) and abnormal (B) PAT were fractionated by two-dimensional isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In autoradiogram B, a protein of 140,000 apparent mol wt and pI of 4.7 is absent but is present in the control platelets shown in A. Mol wt standards are shown on right, and the relative isoelectric points of fractionated polypeptides are shown on the ordinate.

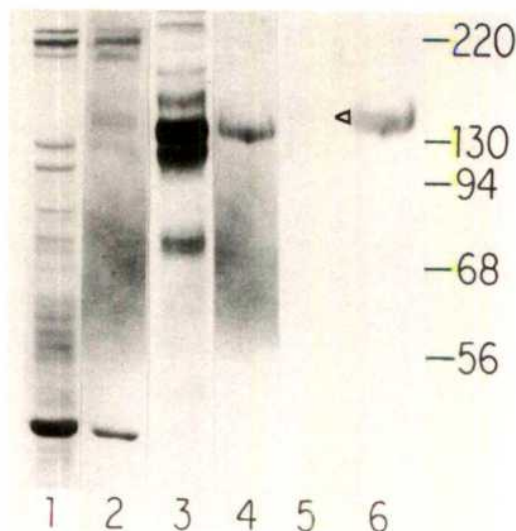


Fig 4. Specificity of anti-human platelet glycoprotein GPIb. Washed normal human platelets were reacted with $^3\text{H}(\text{NaBH}_4)$ after periodate treatment. The ^3H -labeled platelets were extracted with NP-40 and then fractionated by sodium dodecyl sulfate (SDS)-electrophoresis on a 7.5% polyacrylamide resolving gel under reducing conditions. Platelet extract: stained with Coomassie blue (lane 1), Alcian blue (lane 2), after fluorography to detect radiolabeled surface glycoproteins (lane 3), and in a nitrocellulose replica after reaction with the anti-human platelet glycoprotein antiserum, which stained a polypeptide of 140,000 mol wt (Δ) (lane 5). Lanes 4 and 6 represent the corresponding reactions of purified human platelet glycoprotein (3 μg) with Alcian blue and with the antiglycoprotein antiserum, respectively. Positions of mol wt standards are shown on right.

platelets from seven normal control subjects (Fig 5, lanes 3 through 6). Degradation products of GPIb were visible (indicated by low mol wt polypeptide that reacted with the antiglycocalicin antiserum) in four patients with abnormal PAT (Fig 5, lanes 3 and 4). The apparent mol wt for two degradation polypeptides shown by the two diffuse bands in Fig 5, lane 4 were 120,000 and 105,000.

Ristocetin-induced platelet agglutination was significantly lower in patients with abnormal PAT. For the group with normal PAT, the average steepest slope of agglutination was 1.25 ± 0.6 ($n = 28$). For the group with abnormal PAT, the average slope was 0.37 ± 0.34 ($n = 26$) (Fig 6). The difference between those two groups was statistically significant ($P < .001$).

Attempts to reproduce the PAT defect in normal platelets in vitro were made by exposing platelets to xylocaine, which is known to stimulate proteolysis of GPIb.¹⁹ In two experiments, xylocaine treatment produced a decrease of antithrombin activity in platelets. The PAT was reduced from 31 seconds before treatment to 21 seconds after treatment in one experiment and from 23 seconds to 18 seconds in another. The effect of the xylocaine treatment could be seen on the two-dimensional PAGE and autoradiogram of ³H-labeled platelets as an absence of a protein of 140,000 mol wt at pI of 4.5 to 5.2 (Fig 7).

DISCUSSION

Platelet antithrombin activity denotes the ability of platelets to neutralize the clotting activity of thrombin.¹ Previous reports^{21,22} have implicated GPIb as the protein responsible

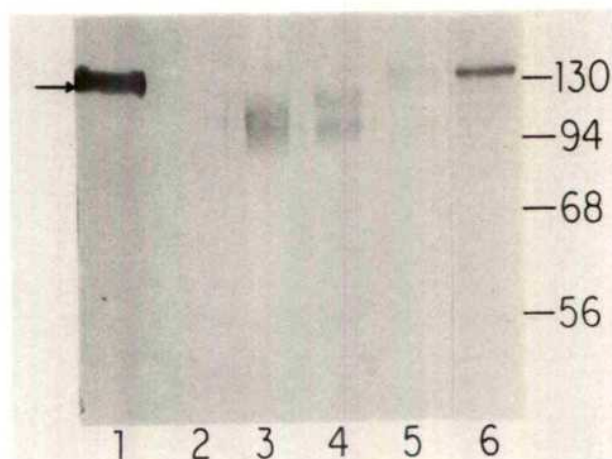


Fig 5. Alterations of the platelet GPIb in patients with abnormal platelet antithrombin test (PAT). Platelets (40 μ g) and purified glycocalicin (2 μ g) and thrombospondin (2 μ g) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to the nitrocellulose paper. The replicas were stained by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG after incubation with a monospecific antiserum against purified human platelet glycocalicin. Lane 1, purified glycocalicin; lane 2, purified thrombospondin; lanes 3 through 5, patients with abnormal PAT; lane 6, healthy control subject. The estimated mol wt for glycocalicin is 127,000 (lane 1); for GPIb (lanes 5 and 6), 140,000, and for the two degradation polypeptides in lane 4, 120,000 and 105,000. Position of standard mol wt proteins is shown on right.

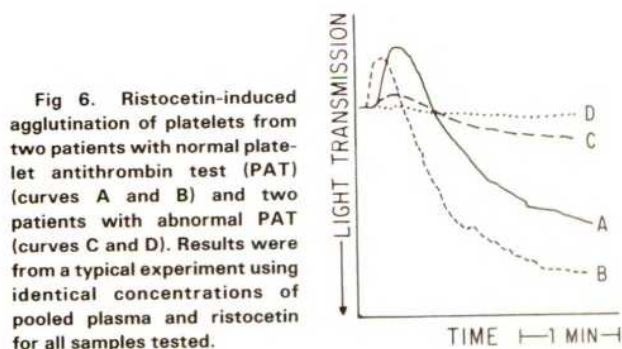


Fig 6. Ristocetin-induced agglutination of platelets from two patients with normal platelet antithrombin test (PAT) (curves A and B) and two patients with abnormal PAT (curves C and D). Results were from a typical experiment using identical concentrations of pooled plasma and ristocetin for all samples tested.

for antithrombin activity. Glycocalicin, a proteolytic product of GPIb, has been shown by other investigators to prolong the thrombin clotting time and inhibit competitively ristocetin-induced platelet aggregation^{22,23}; it has been implicated as the site of thrombin and vWF binding within the GPIb molecule.¹² Recently, Takamatsu and colleagues,²⁴ by chemical cross-linking studies, demonstrated one major thrombin receptor on the platelet surface and showed that the receptor binding is blocked by antiglycocalicin antibody.

In this study, a platelet glycoprotein interacting with antiglycocalicin antibody and having a mol wt of 140,000 and a pI of 4.5 to 5.2 was decreased in patients with an abnormal PAT. The electrophoretic pattern (as described previously), which showed characteristics similar to those of

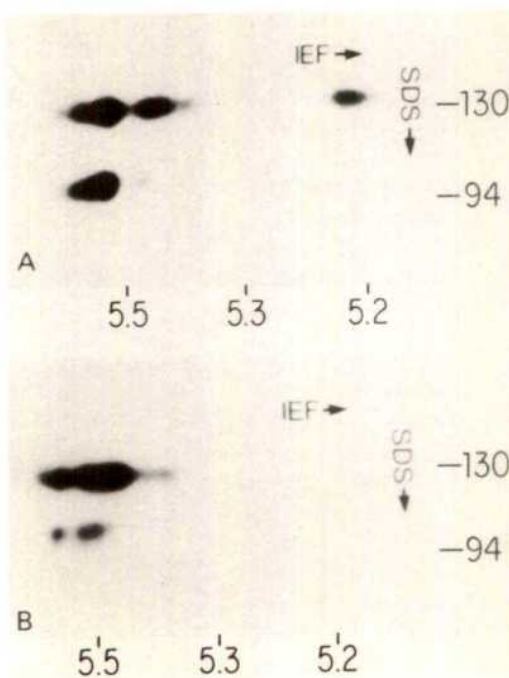


Fig 7. Surface-labeled glycoproteins of platelets after treatment with xylocaine (4 mmol/L). The glycoproteins of xylocaine-treated platelets were ³H-labeled and fractionated by two-dimensional isoelectric focusing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Panel B shows the absence of a protein of apparent mol wt of 140,000 and a pI of 4.5 to 5.2 that is present in the nontreated control platelets (A). Mol wt standards are shown at right, and the relative isoelectric points are shown on the ordinate.

GPIb as reported by others,^{25,26} along with positive staining of the immunoblot by antglycocalicin antibody, suggests that this protein is GPIb.

The mechanism that produces the GPIb defect as demonstrated in this study is not known. It may be due to quantitative reduction of GPIb on the platelet surface or to a structural alteration of the molecule so that it no longer reacts to monospecific antibody against glycocalicin. GPIb is a prominent surface membrane glycoprotein, rich in sialic acid content and, as such, can be readily labeled with tritiated borohydride after generation of reactive carbonyl groups with sodium periodate. When tritiated platelets were analyzed by two-dimensional PAGE, a deficiency of GPIb also was demonstrated in platelets from patients with low PAT. The latter results may reflect less glycosylation of GPIb. Disialation by neuraminidase treatment, however, caused a retardation of electrophoretic mobility of GPIb in SDS-PAGE.²⁷ Results from the present surface labeling and immunoblotting studies failed to show such effects.

Ristocetin-mediated binding of vWF to normal platelets is dependent on the integrity of GPIb.²⁸ Antibodies prepared against GPIb have prevented platelet agglutination in response to ristocetin.²⁹ Platelets of patients with Bernard-Soulier syndrome who are deficient in GPIb also fail to respond to ristocetin and bind less thrombin than do normal patients.³⁰

Decreased concentrations of GPIb have been described in thrombocythemia by other investigators.^{31,32} This is consistent with the previously reported decreases in PAT in that disease.⁴ Additional supporting evidence that GPIb is the platelet protein accounting for most platelet antithrombin activity comes from experiments dealing with exposure of normal platelets to xylocaine. This procedure causes cleavage of glycocalicin from GPIb (Fig 7) as well as diminished platelet antithrombin activity.

Both thrombin and plasmin are known to degrade GPIb.^{23,33} Therefore, the depletion of GPIb from platelets may be the result of the generation of circulating proteolytic enzymes in cancer patients. No direct evidence confirms proteolysis of GPIb of platelets with reduced PAT; however, the demonstration of low mol wt fragments of GPIb is consistent with proteolytic degradation of this surface glycoprotein. Release of calcium-dependent protease (CDP) from platelets can also cause depletion of GPIb.^{28,34} Because all

platelet samples were prepared and lysed in the presence of EDTA and iodoacetamide, which are inhibitors of CDP,³⁵ it is doubtful that depletion of GPIb from platelets is an *in vitro* phenomenon caused by the release of CDP during washing and solubilization of platelets.

Diminished platelet sialic acid content has previously been associated with malignancy,³⁶ as has increased plasma sialic acid.³⁷ The latter has been correlated with the extent of tumor mass.³² Because glycocalicin is found to circulate in normal plasma,³⁸ it would be of interest to determine if any portion of the increased plasma sialic acid is accounted for by glycocalicin.

Decreased amounts of a second protein of apparent mol wt of 180,000 were also found in platelets of patients with low PAT. This protein was identified as thrombospondin on the basis of a comparison of its electrophoretic, immunologic, and staining properties with those of purified thrombospondin. Thrombospondin is an α -granular protein, composed of three apparently identical chains. It is released and expressed on the platelet surface in response to thrombin stimulation.¹⁵ Thrombospondin is deficient in platelets from patients with gray platelet syndrome³⁹ as well as from patients with thrombocythemia.^{40,41} Thrombospondin has been postulated to have multiple functions. The presence of thrombospondin on the platelet membrane was found to increase fibrinogen binding.⁴² Because surface-bound fibrinogen accounts for 25% of the platelet antithrombin activity,³ the absence of thrombospondin could indirectly account for the decrease in PAT.

It is unknown at this time whether the similar protein changes account for platelet antithrombin defect in nonmalignant disease.⁴ In addition, the unequivocal proof of a cause-effect relationship between platelet antithrombin deficiency and the depletion of GPIb and thrombospondin is still lacking. Nevertheless, this study provides the first evidence to link the antithrombin defect and alterations of glycoproteins in platelets from patients with malignant disease.

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Interrelationship Between Mitosis and Endomitosis in Cultures of Human Megakaryocyte Progenitor Cells

By Moises Arriaga, Karen South, Janet Lee Cohen, and Eric M. Mazur

Sera from dogs rendered aplastic by total-body irradiation stimulate human bone marrow megakaryocyte progenitors to form megakaryocyte colonies in plasma clot cultures. In this investigation, we evaluated the effects of varying concentrations of such sera on both the mitotic and endomitotic phases of human megakaryocyte development in vitro. When low concentrations of aplastic canine sera (2.5% to 5.0% [vol/vol]) were added to cultures of human peripheral blood mononuclear cells in place of normal AB serum, megakaryocyte colony formation was augmented fivefold, cell numbers per colony increased approximately 2.5-fold, and the geometric mean megakaryocyte ploidy almost doubled. Further increasing the aplastic canine serum concentration from 10% to 30% (vol/vol) stimulated no additional colony formation. However, there was a further augmentation of cell numbers per colony associated with a progressive decrease in the mean megakaryocyte ploidy. Megakaryocyte cultures were harvested after 7, 12, 15, and 19 days of incubation, and these demonstrated that the lower mean ploidy values found at

the higher concentrations of aplastic canine serum did not result from delayed endoreduplication. At all aplastic serum concentrations evaluated, there existed a strong correlation between nuclear ploidy and cell diameter. We conclude that both the mitotic and endomitotic events in human megakaryocytopoiesis may be influenced by a factor or factors present in aplastic canine serum. At lower in vitro concentrations, such sera stimulate both mitosis and endomitosis, which promotes the development of megakaryocyte colonies composed of larger cells with a higher mean ploidy. With increasing aplastic serum concentrations, colony formation plateaus and mitosis is favored over endomitosis. This results in colonies composed of more numerous but smaller megakaryocytes with a lower mean ploidy. Our data suggest that the size and extent of polyploidization that can be achieved by a developing megakaryocyte may be influenced by the mitotic prior history of its immediate precursor cell.

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THE DEVELOPMENT of a clonogenic in vitro culture system for the human megakaryocyte progenitor cell (CFU-Meg) has led to the identification of a serum substance, megakaryocyte colony-stimulating factor (Meg-CSF), that stimulates mitosis and the early development of the CFU-Meg.¹⁻³ This factor promotes in vitro megakaryocyte colony formation from normal human CFU-Meg and increases the number of megakaryocytes developing in each colony.¹ Meg-CSF has been detected in sera obtained from patients with aplastic anemia,^{1,2} amegakaryocytic thrombocytopenia⁴ and from patients who are at a hematologic nadir following chemotherapy.⁵ Because Meg-CSF is not detected in sera from patients with consumptive thrombocytopenia, we have postulated that its elaboration is a response to the depletion of bone marrow megakaryocytes or megakaryocyte progenitor cells.²

We recently described and partially characterized an analogous Meg-CSF in the sera of dogs rendered aplastic by total-body irradiation.⁶ Canine is as active as human Meg-

CSF in promoting mitotic expansion and the early development of human megakaryocyte progenitors. However, resultant platelet production is determined not only by the number of cell divisions occurring during CFU-Meg development. The mitotic amplification of each megakaryocyte progenitor cell is generally followed by a variable number of endomitotic nuclear replications. This results in a population of mature megakaryocytes that is heterogeneous with respect to ploidy. Because platelet production per megakaryocyte increases with ploidy, the total number of platelets produced from the development of each megakaryocyte progenitor is determined predominantly by the extents to which mitosis and endomitosis occur.⁷

The regulatory determinants of megakaryocyte polyploidization are not well understood. In this investigation, we have explored the hypothesis that mitotic events occurring during early megakaryocytopoiesis may interact with subsequent megakaryocyte polyploidization. We have used a plasma clot culture system for human peripheral blood CFU-Meg in which varying mitotic stimulation was provided by different concentrations of Meg-CSF containing aplastic canine sera. Techniques were developed for the concurrent in situ immunohistochemical identification, enumeration, and DNA quantitation of cultured megakaryocytes. Aplastic canine sera induced significant concentration-dependent effects on both the mitotic and endomitotic phases of human megakaryocyte development in vitro. Our data suggest that these two processes may be linked.

MATERIALS AND METHODS

Human subjects. Peripheral blood for CFU-Meg cultures and normal AB sera was obtained by routine venipuncture from consenting healthy adult donors who provided written informed consent.

Canine sera. Aplastic canine sera were prepared as previously described.⁶ The lot of serum used in this investigation was harvested from an animal whose platelet count was 4,000/ μ L at the time of sacrifice.

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Plasma clot megakaryocyte cultures. Human peripheral blood was diluted 1:1 with α -medium minus nucleosides (GIBCO, Grand Island, NY) containing preservative-free heparin at 20 U/mL. Mononuclear cells were isolated by Ficoll 400–sodium diatrizoate (LSM, Litton Bionetics, Kensington, MD) density centrifugation. The cells were suspended in α -medium containing 20% AB serum, and the plastic-adherent cells were removed by a one-hour incubation at 37 °C in 100-mm polystyrene tissue culture dishes (Corning Glass Works, Corning, NY). Plasma clot megakaryocyte cultures were established as previously described.^{1,2,8} Plastic-adherent cell-depleted mononuclear cells were cultured at 10^6 cells/mL in 1-mL volumes in 35-mm Petri dishes. Aplastic canine serum containing Meg-CSF was added to the cultures at time 0 in concentrations ranging from 0% to 30% (vol/vol), replacing equal volumes of normal AB sera in the cultures. A single lot of aplastic canine serum and lots of equivalent normal human AB sera were used in all experiments. Culture dishes were incubated for 12 days (unless otherwise indicated) at 37 °C in a 100% humidified atmosphere of 5% CO₂ in air using a NAPCO 5200 water-jacketed incubator (National Appliance Co, Portland, OR). Harvesting was performed by fixation in situ with methanol:acetone (1:1) for 20 minutes, sequential washing with 0.01 M/L phosphate-buffered saline (PBS), pH 7.2, and distilled water, and then air drying. The plasma clots were stored frozen at –20 °C until immunofluorescent staining was performed.

Immunofluorescent staining and scoring of cultures. Rabbit antiserum to platelet glycoproteins (PGP) was prepared and its specificity demonstrated as previously reported.^{1,2,8} Megakaryocyte colonies were labeled immunofluorescently by sequential incubations with PGP antiserum diluted 1/200 in PBS and rhodamine-conjugated goat antirabbit IgG (IgG fraction) diluted 1/60 in PBS (Cappel Laboratories, Cochranville, PA). A rhodamine rather than fluorescein conjugate was used for the second label because its optical signal was stable to the acid hydrolysis required for DNA quantitation.

After air-drying, the plasma clot megakaryocyte cultures were scored in situ. The 35-mm Petri dishes were inverted, the base areas completely scanned, and rhodamine-positive colonies enumerated with a fluorescent light microscope at 100 $\times$ (Olympus BH-2 microscope with a vertical fluorescent illuminator, Olympus Corp, New York). A megakaryocyte colony was defined as a cluster of three or more intensely rhodamine-positive cells. All experiments were performed in duplicate.

DNA quantitation. Staining for quantitative DNA determination was performed using a modified Feulgen technique adapted from Ruch.⁹ The compound 2,5-bis-(4-aminophenyl)-(1') = 1,3,4 oxadiazole (I), (BAO; Fluka Chemical Corp, Happaug, NY) was chosen as the DNA fluorochrome because its relative stability to UV irradiation resulted in a minimal influence of the time of light exposure on DNA measurements.⁹ BAO is comparable to Auramine O in its specificity for DNA⁹ and has been recommended by Paulus et al for the Feulgen staining of human bone marrow megakaryocytes.¹⁰ In our laboratory, Feulgen/BAO staining is 96% DNA specific based upon the reduction in the nuclear fluorescent signal induced by exposure to deoxyribonuclease I (Sigma Chemical Co, St Louis) prior to staining. For this determination, human peripheral blood smears were fixed in methanol:acetone (1:1), and the test smears were preincubated in DNAase I at 0.5 mg/mL in 0.03 M/L magnesium sulfate, pH 6.0. Control and test smears were Feulgen stained with BAO as will be described and the mean nuclear fluorescent signals determined from the nuclei of polymorphonuclear leukocytes. Following DNA digestion, the mean nuclear fluorescence was reduced from 49.2 to 2.0 (arbitrary units).

The fixed, PGP-rhodamine-labeled plasma clot cultures were rehydrated with distilled water, and then the DNA was hydrolyzed

in 4 N HCl at 37 °C for 20 minutes. Hydrolysis was terminated by aspirating the HCl and flooding the culture dishes with distilled water at room temperature. Nuclear DNA was stained using freshly prepared BAO reagent (20 parts 0.01% BAO in distilled water (wt/vol) to two parts 1 N HCl to one part 10% sodium metabisulfite [wt/vol]). One milliliter of BAO reagent was layered on each 35-mm Petri dish, and the cultures were gently agitated for 30 minutes at room temperature in a 100% humidified chamber. BAO staining was followed by three two-minute washes with sulfite water (18 parts distilled water, one part 1 N HCl, one part 10% sodium metabisulfite [wt/vol]), one ten-minute wash with distilled water, and dehydration with 70% ethanol. The culture dishes were air-dried, the plastic sides removed, and circular coverslips mounted with glycerol.

Megakaryocyte DNA quantitation was performed using a Leitz Laborlux 12 microscope equipped with an adjustable measuring diaphragm, an MPV compact microscope photometer, and interchangeable filter cubes (Ernst Leitz, Wetzlar, W Germany) interfaced with an Epson HX-20 computer (Epson America, Inc, Torrance, CA). The measuring diaphragm was located in the emitted light pathway, and adjustment of its aperture did not influence incident illumination.⁹ Green-band "Cube N2" (excitation filter BP 530 to 560 nm and barrier filter LP 580 nm) was used for the identification and enumeration of the PGP-rhodamine-labeled megakaryocytes, and wide-band UV "Cube A" (excitation filter BP 340 to 380 nm and barrier filter LP 430 nm) was used to measure BAO fluorescence.

At a magnification of 100 $\times$ the megakaryocyte colonies were identified in situ in the plasma clot by rhodamine fluorescence, and single megakaryocytes were randomly selected for DNA quantitation. When the total number of megakaryocytes in a culture plate exceeded 200, representative sampling was assured by (1) measuring nuclear DNA in equal numbers of cells from both halves of that culture plate and (2) analyzing no more than three to four arbitrarily selected megakaryocytes in each colony. Within the colonies, cells were chosen based upon the order in which they were encountered while scanning using green incident light at 400 $\times$. This sampling algorithm was validated in ten representative instances by comparing repeat ploidy distributions determined each from 100 randomly chosen megakaryocytes in the same culture plate. Ploidy distributions did not differ significantly between paired determinations, and duplicate mean ploidy values had coefficients of variation averaging $8.6\% \pm 5.9\%$ ($\pm$ SD).

Megakaryocyte DNA was measured using UV light excitation. At a magnification of 400 $\times$ the nucleus of each megakaryocyte was isolated using the adjustable measuring diaphragm, and its BAO fluorescent signal was quantitated photometrically. The background readings were taken in the same high-power field as each megakaryocyte using the same diaphragmatic aperture. The net megakaryocyte DNA emission signal (M_i) was calculated as the difference of the two values. Readings of diploid-cell DNA fluorescence, D_j , (corrected for the background) were obtained throughout the culture specimen in a diploid cell:megakaryocyte ratio of approximately 1:2. When a culture dish was completely scored, the relative DNA content per cell for each of the megakaryocytes was determined using the following relationship:

$$\text{Megakaryocyte DNA content (i)} = \frac{M_i}{\left[\sum_{j=1}^n D_j \right]} \times 2$$

where n was the number of diploid cells evaluated in that culture dish. Megakaryocytes were assigned to the ploidy level of the nearest modal value of DNA content according to the algorithm of Paulus et al.¹⁰ The measurements, calculations, and ploidy assignment were

performed using an on-line Epson HX-20 computer that permitted the evaluation of 75 to 100 cells per hour. Each high-power field received a total of no more than 30 seconds of UV light exposure.

The mean megakaryocyte ploidy was calculated from the following equation:

$$\text{Mean megakaryocyte ploidy} = \text{anti-log}_2 \left[\frac{\sum_{p=2}^{64} \log_2(p) \times n_p}{n} \right]$$

where n_p is the number of megakaryocytes in ploidy class p ($p = 2, 4, 8, \dots, 64$) and n is the combined number of megakaryocytes in all ploidy classes. The geometric mean megakaryocyte ploidy is a more accurate representation of the mean ploidy than the linear average used in some studies^{11,12} because the linear average disproportionately weights the contribution of higher-ploidy megakaryocytes.

Megakaryocyte size. Megakaryocyte diameters were determined at 400 $\times$ magnification using a calibrated eyepiece micrometer.

Statistical analyses. Megakaryocyte ploidy distributions were compared by Pearson's χ^2 test of independence of distributions.¹³ Other comparisons were made using Student's t test or linear regression analyses.

RESULTS

Similar to what we have reported previously,⁸ low-level megakaryocyte colony formation from peripheral blood mononuclear cells was observed in unstimulated cultures containing only normal human AB serum. In such cultures 10.4 ± 3.8 (mean $\pm$ SEM) megakaryocyte colonies were grown from 10^6 mononuclear cells (Fig 1). The addition of aplastic canine serum stimulated up to a fivefold increase in colony formation (Fig 1). The megakaryocyte colony numbers reached a maximum of 54.4 ± 11.2 (mean $\pm$ SEM) at an aplastic canine serum concentration in a culture of 2.5% (vol/vol). With further increases in the aplastic canine serum

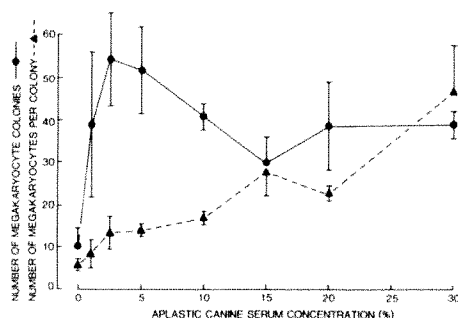


Fig 1. Effects of the concentration of aplastic canine serum on megakaryocyte colony numbers (●) and the number of megakaryocytes per colony (▲) after 12 days in culture. Each point represents the mean $\pm$ SEM of three (cells/colony) or four (colony numbers) separate experiments where assays were performed in duplicate. The data on megakaryocytes per colony incorporated observations from a total of 866 colonies whose distribution by size is displayed in Table 1. Megakaryocyte colony numbers were significantly increased ($P < .05$ by Student's t test) above the control (0%) at all aplastic canine serum concentrations evaluated except 1%. Colony numbers were maximal at 2.5% aplastic canine serum, and the numbers observed between 5% and 30% did not differ significantly from the peak value. The mean number of megakaryocytes per colony was linearly related to the concentration of aplastic canine serum ($r = .952$, $P < .001$).

concentration, the megakaryocyte colony formation plateaued (Fig 1).

Aplastic canine serum also enhanced the number of cells developing in each megakaryocyte colony, but there was no plateau in the dose-response effect. The mean number of megakaryocytes in each colony increased almost monotonically throughout the entire concentration range of aplastic canine serum evaluated (Fig 1). Cells per colony rose from 5.8 ± 0.5 (mean $\pm$ SEM) in cultures containing only normal AB serum to 46.1 ± 11.4 (mean $\pm$ SEM) at the maximum aplastic canine serum concentration of 30% (vol/vol). The distribution of megakaryocyte colony size as a function of aplastic canine serum concentration is presented in Table 1. With increasing aplastic canine serum concentrations, there was a continuous upward shift in the modal colony size. In addition, the maximal colony sizes progressively increased, which reflected the stimulation of up to four mitotic generations beyond that observed in unstimulated cultures.

The same cultures used to evaluate colony numbers and size were stained with BAO for DNA quantitation. Because individual megakaryocyte colonies grown in plasma clot cultures are typically dispersed,^{1,8} overlapping of constituent cells was not a significant problem in performing such measurements. Illustrated in Fig 2 is a representative megakaryocyte colony in which both megakaryocyte cytoplasm is labeled immunochemically with anti-PGP/rhodamine and nuclei are stained with BAO. In Fig 2B there are additional cells whose nuclei stain with BAO but do not label with the anti-PGP/rhodamine. These cells, consisting predominantly of macrophages and lymphocytes, served as the diploid standards from which megakaryocyte ploidy was calculated. The coefficient of variation for the diploid cell DNA fluorescent signals averaged 32.5%, and the background fluorescence constituted approximately 30% of the total 2N nuclear measurements.

The effect of increasing concentrations of aplastic canine serum on cultured megakaryocyte mean ploidy was biphasic (Fig 3). Between aplastic serum concentrations of 0% and 2.5% (vol/vol), a significant enhancement of ploidy was observed, with the mean values rising from 6.97N at 0% to 12.66N at 2.5% ($P < .01$ by Pearson's χ^2).¹³ The peak megakaryocyte ploidy was observed at the same aplastic serum concentration as that at which colony formation attained its maximum (Fig 1). The mean ploidy decreased with further increases in the aplastic serum concentration and reached a minimum of 4.98N at 30% aplastic serum. The enhancement of megakaryocyte ploidy by aplastic serum at concentrations of 2.5% to 10% was a result of a shift in the distribution of cells from the 4N and 8N to the 16N and 32N ploidy classes. At aplastic canine serum concentrations of 15% to 30% in culture, a decreasing mean ploidy resulted from increases in 2N and 4N cells at the expense of 16N, 32N, and 64N cells (Table 2).

The kinetic aspects of megakaryocyte polyploidization in culture were determined to evaluate the impact of culture duration on ploidy. Megakaryocyte cultures containing 0%, 2.5%, and 20% aplastic canine serum were harvested serially following 7, 12, 15, and 19 days of incubation (Fig 4). The peak mean ploidy values were observed on days 12 to 15 of

Table 1. Distribution of Megakaryocyte Colony Size as a Function of the Concentration of Aplastic Canine Serum in Culture

Aplastic Canine Serum Concentration (%) [*]	No. of Megakaryocyte Colonies Evaluated	Mean Number of Mitotic Generations† (Megakaryocytes/Colony—Range)						
		2 (3–5)	3 (6–10)	4 (11–22)	5 (23–44)	6 (45–90)	7 (91–180)	8 (181–361)
0	30	63.3	23.3	13.3	0	0	0	0
1	54	29.6	33.3	27.8	9.3	0	0	0
2.5	143	29.4	28.0	26.6	14.7	1.4	0	0
5	140	12.1	37.1	34.3	13.6	2.9	0	0
10	147	8.2	27.2	42.9	17.7	4.1	0	0
15	107	7.5	15.0	32.7	28.0	9.4	7.5	0
20	155	8.4	24.5	32.3	23.9	10.3	0.7	0
30	90	7.8	13.3	25.6	25.6	11.1	10.0	6.7

The data are derived from three experiments and a total of 866 colonies. The arithmetic means and statistical analysis of these data are presented in Fig 1.

*The concentration (vol/vol) of aplastic canine serum in culture replacing an equal volume of normal AB serum.

†The interval range for colony size is calculated as $2^{(n-0.5)}$ to $2^{(n+0.5)}$ ($n = 2, 3, \dots, 8$), where n represents successive mitotic generations (doublings). The values are given as the percentage of the total colonies.

culture regardless of the concentration of aplastic serum used. The biphasic relationship of mean ploidy with aplastic serum concentration was seen as early as seven days and persisted through day 15 of culture. By day 19, many of the megakaryocyte colonies had degenerated, and the mean ploidies became equivalent at all serum concentrations tested. In the cultures containing 20% aplastic canine serum, there was no increase in ploidy beyond 12 days of culture. Thus, the lower mean ploidy seen in day 12 cultures (Fig 3) was not simply the result of delayed endoreduplication.

Because megakaryocyte size is known to vary with ploidy *in vivo*,^{7,14} this relationship was analyzed in the *in vitro* culture system. At all aplastic canine serum concentrations evaluated, the megakaryocyte size varied significantly with $\log_2$ (ploidy). The slopes and intercepts of the regression lines relating these two parameters did not vary significantly among the different aplastic serum concentrations (data not shown). Therefore, megakaryocyte size *v* ploidy data from all aplastic canine serum concentrations were consolidated and are presented in Fig 5.

DISCUSSION

The production of platelets from the committed megakaryocyte progenitor cell or CFU-Meg is complex, requiring

nearly sequential mitosis, endomitosis, and cytoplasmic maturation.⁷ However, the physiological regulatory control systems governing these processes are for the most part poorly understood. We have recently reported that sera from patients with bone marrow megakaryocyte aplasia^{1,2,4} and from dogs with irradiation-induced aplasia⁶ contain a substance, Meg-CSF, that stimulates mitosis and the early development of human bone marrow CFU-Meg. Meg-CSF manifests this effect in culture by augmenting both the number of megakaryocyte colonies grown and the number of component cells developing in each colony.^{1,3}

In this report, we establish that Meg-CSF containing aplastic canine serum has qualitatively similar effects on human peripheral blood CFU-Meg progenitors. We have also been able to demonstrate an interaction between varying mitotic stimulation of early megakaryocytopoiesis by aplastic serum and the degree of megakaryocyte polyploidization achieved in culture.

We observed an unexpected biphasic effect of increasing concentrations of aplastic canine serum on *in vitro* megakaryocytopoiesis. Compared with control cultures containing only normal AB serum, low culture concentrations of aplastic canine serum (2.5% to 5.0% [vol/vol]) augmented megakaryocyte colony formation fivefold, increased cell numbers

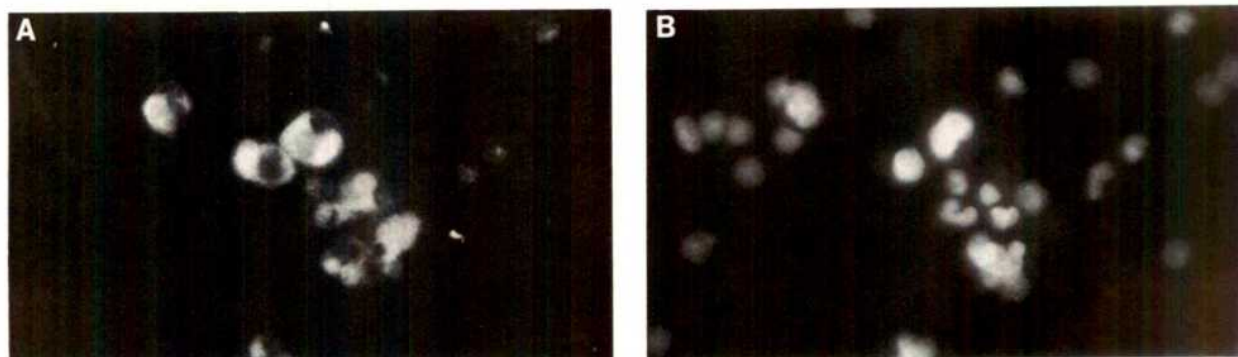


Fig 2. Representative megakaryocyte colony double labeled with PGP antiserum/rhodamine (A, green incident light) and BAO (B, UV incident light) that was cultured from human peripheral blood mononuclear cells in the presence of aplastic canine serum (original magnification $\times 100$).

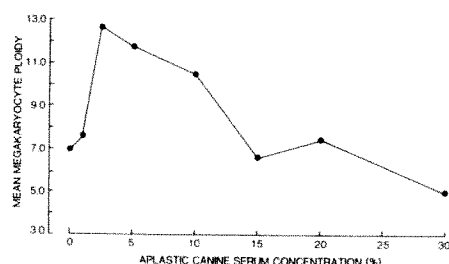


Fig 3. Effect of the concentration of aplastic canine serum in culture on the geometric mean ploidy of megakaryocytes cultured from human peripheral blood mononuclear cells. Data from three experiments were combined and include ploidy determinations on a total of 2,078 megakaryocytes. A mean of 260 cells was evaluated at each point (Table 2). Ploidy distributions at adjacent aplastic canine serum concentrations differ significantly in the following instances: 1.0% v 2.5%, 10% v 15%, and 20% v 30% ($P < .01$ by Pearson's χ^2); and 5% v 10% ($.01 < P < .02$).

per colony approximately 2.5-fold, and almost doubled the mean ploidy. Further increasing the aplastic serum concentration from 10% to 30% (vol/vol) stimulated no additional colony formation. However, there was a further augmentation of cell numbers per colony associated with a progressive decrease in the mean megakaryocyte ploidy. Thus, in those cultures containing the higher concentrations of aplastic canine serum, an inverse relationship was present between mitotic amplification of the megakaryocyte progenitor (as manifested by cell numbers per colony) and the extent of megakaryocyte endoreduplication.

Our results appear to conflict with similar studies in mice reported by Chatelain and Burstein.¹⁵ They found no effect of increasing concentrations of pokeweed mitogen-stimulated spleen conditioned medium on cultured murine megakaryocyte ploidy, despite slight augmentations in colony numbers and size. The effects that we observed using aplastic canine serum may therefore be dependent upon this specific

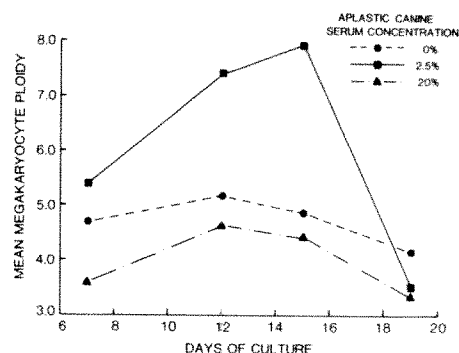


Fig 4. Effect of culture duration on the geometric mean ploidy of megakaryocytes cultured from human peripheral blood mononuclear cells. The cultures contained aplastic canine serum concentrations of 0% (●), 2.5% (■), and 20% (▲). Data are derived from two experiments with ploidy determinations on 3,163 megakaryocytes.

source of Meg-CSF, result from the higher cell plating densities we used, or reflect the substantially greater changes in colony size seen in our studies.

That megakaryocyte development from its progenitor may be characterized by a reciprocal relationship between mitosis and endomitosis has been demonstrated previously by other investigators.^{11,15-17} However, these earlier studies analyzed differences among individual murine megakaryocyte colonies that were generally cultured under conditions providing maximal growth stimulation. It was found that colonies with larger numbers of cells had lower mean ploidy values. Cell number and mean ploidy differences among colonies were attributed to an intrinsic heterogeneity of the CFU-Meg progenitor cell population.

Our studies indicate that, although CFU-Meg heterogeneity may exist, mitosis and endomitosis can be further influenced in culture by a factor or factors present in aplastic canine serum. Because colony numbers plateau at aplastic serum concentrations of 2.5% and above, it is probable that the higher aplastic serum concentrations affect a fixed population of megakaryocyte progenitors rather than selectively recruit different progenitor classes. Therefore, the

Table 2. Ploidy Distributions of Progenitor Cell—Derived Megakaryocytes Cultured With Varying Concentrations of Aplastic Canine Serum

Aplastic Canine Serum Concentration (%) [*]	No. of Megakaryocytes Evaluated	Ploidy Class [†]					
		2N	4N	8N	16N	32N	64N
0	111	4.5	27.0	35.1	20.7	7.2	5.4
1	92	7.6	22.8	46.7	15.2	7.6	0
2.5	275	4.7	13.8	24.4	31.6	18.5	6.9
5	375	6.4	12.8	28.8	28.3	18.4	5.3
10	348 [‡]	6.3	19.0	27.9	27.6	14.9	4.0
15	364	19.2	24.5	29.9	19.2	6.9	0.3
20	323	12.7	23.2	33.7	22.9	7.4	0
30	190	28.9	28.9	27.4	11.6	2.6	0.5

The data represent three experiments and a total of 2,078 megakaryocytes. The geometric means and statistical analyses of these data are presented in Fig 3.

^{*}The concentration (vol/vol) of aplastic canine serum in culture replacing an equal volume of normal AB serum.

[†]The values are given as a percentage of the total cells.

[‡]One cell (0.3% of total) classified as 128N.

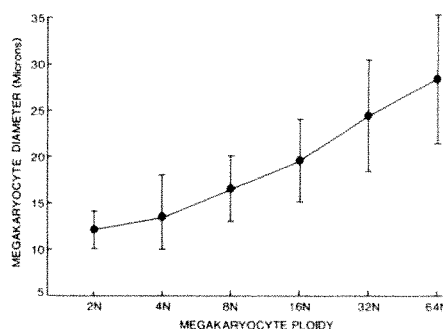


Fig 5. Relationship between diameter and ploidy class for megakaryocytes cultured from human peripheral blood mononuclear cells. Data are derived from 715 cells and are presented as the mean $\pm$ SD. A significant linear correlation was observed ($r = .665$, $P < .001$).

inverse relationship between mitosis and endomitosis that was previously attributed exclusively to progenitor cell heterogeneity likely also characterizes megakaryocytopoiesis in general. It is speculative whether this relationship is physiologically adaptive and operates *in vivo*. However, one may hypothesize that in conditions of severe megakaryocytic hypoplasia with accompanying thrombocytopenia, mitotic amplification of existing megakaryocyte precursors, even at the expense of endomitosis, may most effectively restore platelet production. This hypothesis is supported by a recent report by Tanum who demonstrated that the resumption of platelet synthesis in rats receiving a sublethal dose of thiopeta is temporally associated with the exclusive appearance of low-ploidy megakaryocytes in the bone marrow.¹⁸

Although Meg-CSF is known to be present in aplastic canine sera, this investigation does not distinguish between its effects and those of other regulators potentially present in such sera. The existence of additional regulators that influence megakaryocyte endoreduplication and cytoplasmic maturation have been postulated by several investigators.¹⁹⁻²² However, even if these exist, our data indicate that their influence may be limited by the mitotic history of the cells upon which they act. Another hypothesis consistent with our data is that Meg-CSF alone controls both the mitotic and endomitotic phases of megakaryocytopoiesis, with differential effects depending upon the intensity of Meg-CSF stimulation.

The normal AB sera used in our experiments may also have included factors capable of affecting megakaryocyte development *in vitro*. Several investigators have suggested that normal sera contain platelet-derived inhibitors of megakaryocyte colony formation.^{23,24} However, it is unlikely that such inhibitors, if present, substantially influenced our experimental results because both peak megakaryocyte colony formation and maximal nuclear polyploidization were achieved at a concentration ratio of aplastic canine to normal AB serum of less than 0.10. It is also improbable that the aplastic canine serum contained substantial quantities of platelet-derived inhibitors, given the low platelet count at the time of its preparation. We have recently established that the megakaryocyte colony-stimulating activity manifested by aplastic canine sera results from the presence of a positive regulatory protein rather than from the absence of inhibitors found in normal sera.⁶

By examining the polyploidization of human megakaryocyte progenitors in culture, our studies represent an extension of the previously published murine *in vitro* studies.^{11,12,15-17,22} Our use of immunochemical labeling of cultured human megakaryocyte colonies for identification with concurrent

Feulgen-BAO staining *in situ* for DNA quantitation is analogous to those murine studies in which megakaryocyte colonies were double labeled *in situ* for acetylcholinesterase and DNA.¹⁵⁻¹⁷ Such approaches provide unequivocal identification of all megakaryocytes present within a culture (including low-ploidy cells) and eliminate both the biases inherent in morphological identification using inverted microscopy and the cell losses that accompany colony transfer to glass slides for DNA analysis. The use of Feulgen-BAO staining coupled with microfluorometry for the determination of the cellular DNA content represents a technically straightforward and accurate approach to DNA quantitation, one that does not require the expensive equipment necessary for scanning microdensitometry. Furthermore, distributional errors, excessive signal-to-noise ratio, and light scatter are minimized or eliminated.^{10,25} Imprecision resulting from photodecomposition of the DNA fluorochrome is minimized by using BAO for which the fluorescent signal is relatively stable to the UV incident light used for excitation.⁹

This investigation also examined the relationship between cultured megakaryocyte ploidy and diameter. The positive correlation we observed (Fig 5) is similar to that previously described for megakaryocytes harvested directly from bone marrow.¹⁴ Our data defined a smaller megakaryocyte diameter for each ploidy class than that described by these other investigators. Possible explanations for this discrepancy include cell shrinkage due to fixation or the failure of the cultured megakaryocytes to achieve full cytoplasmic maturation *in vitro*. Nevertheless, this positive correlation demonstrates that the link between ploidy and cytoplasmic development previously found to exist *in vivo* is maintained during *in vitro* megakaryocyte development.

The availability of megakaryocyte progenitor cell culture systems in which one can concurrently quantify colony formation, cell numbers per colony, and endoreduplication provides new opportunities for analyses of megakaryocytopoietic regulation. Our data indicating that the mitotic history of a megakaryocyte precursor may influence its endoreduplicative potential serve to emphasize both the complexity of this regulatory system and the potential utility of the methods described in this investigation.

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The Role of Glutathione Reductase in Maintaining Human Granulocyte Function and Sensitivity to Exogenous H_2O_2

By Harvey J. Cohen, Elizabeth H. Tape, John Novak, Margaret E. Chovaniec, Patricia Liegey, and John C. Whitin

Human granulocytes (polymorphonuclear leukocytes, PMN) produce H_2O_2 and other reactive oxygen species while undergoing phagocytosis. To examine the role of the glutathione cycle in metabolizing H_2O_2 , we incubated PMN with 1,3-bis(2-chloroethyl) nitrosourea (BCNU). Incubation of PMN with BCNU results in a dose-dependent inhibition of PMN glutathione reductase (GRED), with 50% inhibition occurring at approximately 2 $\mu\text{g}/\text{mL}$ BCNU. PMN hexose monophosphate shunt activity stimulated with an exogenous H_2O_2 -generating system was inhibited only when the GRED activity was reduced to less than 30% of control. BCNU-treated cells contained lower levels of reduced sulfhydryls and reduced glutathione, which decreased even more in the presence of an exogenous H_2O_2 -generating system. The effect of BCNU and exogenous H_2O_2 on various aspects of phagocytosis were examined. Exposure of

BCNU-treated PMN to an H_2O_2 -generating system resulted in an inhibition of chemotactic peptide-induced shape changes and degranulation. The ability of BCNU-treated cells to produce O_2^- was diminished only when the PMN were incubated with an H_2O_2 -generating system in the presence of cyanide. Ingestion of opsonized bacteria by BCNU-treated PMN was unaffected by incubation in an H_2O_2 -generating system even in the presence of cyanide. We conclude that (1) PMN GRED is inhibited by BCNU, (2) the ability of PMN to metabolize H_2O_2 is affected only when GRED is reduced more than 70%, (3) this inhibition affects the glutathione content of these cells, and (4) some, but not all of the phagocytic functions of GRED-inhibited PMN are inhibited after exposure to an H_2O_2 -generating system.

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GRANULOCYTES (polymorphonuclear leukocytes, PMN) are thought to effect their bactericidal function through a respiratory burst¹ that results in the generation of reactive oxygen species²⁻⁴ mediated by the activity of a membrane-bound NADPH-dependent oxidase system.^{5,6} The reactive oxygen species may be toxic to other cells as well as to some aspects of PMN function.^{7,8} H_2O_2 has been implicated as one of the possible reactive oxygen species toxic to PMN function.^{8,9}

Jandl et al found a substantial increase in the oxidase activity if PMN were exposed to a stimulus under anaerobic conditions or in the presence of inhibitors of myeloperoxidase.¹⁰ Selenium-deficient rat PMN with diminished glutathione peroxidase activity manifested an increased sensitivity of their O_2^- generating system to either exogenous or endogenous H_2O_2 .¹¹ PMN of patients with a complete glutathione reductase (GRED) deficiency demonstrated an abbreviated oxidative burst with a greater and more rapid fall in reduced sulfhydryl content than normal PMN.¹²

The observation that 1,3-bis(2-chloroethyl)-nitrosourea (BCNU) selectively inhibits cellular GRED^{13,14} has permitted us to examine in depth the effects of GRED on PMN function. We have examined the effects of BCNU on PMN GRED activity, hexose monophosphate shunt (HMPS) activity, reduced sulfhydryl and glutathione content, and various aspects of phagocytic function in the presence and absence of an external source of H_2O_2 . This report describes the variable sensitivity of PMN function to BCNU-mediated inhibition of GRED.

MATERIALS AND METHODS

Materials

Ferricytochrome c type VI, superoxide dismutase (SOD), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), flavine adenine dinucleotide (FAD) NADPH, reduced glutathione (GSH), oxidized glutathione (GSSG), L-leucine, Concanavalin A type IV (ConA), N-formyl-methionylleucylphenylalanine (FMLP), *p*-nitrophenyl β -D-glucuronide, *o*-phthalaldehyde (OPT), methylene blue, N-ethyl maleimide (NEM), tertiary butyl hydroperoxide (t-BuOOH), nitroblue tetrazolium (NBT), glucose oxidase type X (GO), and L-amino acid

oxidase type V (LAAO) were purchased from Sigma Chemical Co, St Louis; dextran T-500 and Ficoll-Paque from Pharmacia Fine Chemicals, Piscataway, NJ; BCNU from Bristol Laboratories, Syracuse, NY; phytohemagglutinin (PMA) from Consolidated Midland Corp, Brewster, NY; zymosan from ICN Pharmaceuticals, Cleveland; cytochalasin B from Aldrich Chemicals, Milwaukee; and $1-^{14}\text{C}$ -glucose, $U-^{14}\text{C}$ -glucose and $U-^{14}\text{C}$ -L-leucine from New England Nuclear, Boston. All other reagents were obtained at the highest commercially available grade and used without further purification.

Preparation of PMN

Human peripheral blood PMN were prepared by dextran sedimentation and Ficoll-Paque centrifugation as previously described.¹⁵ The cells were kept at 2 to $5 \times 10^7/\text{mL}$ in phosphate-buffered saline (PBS: 135 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L sodium phosphate buffer, pH 7.4) with glucose (5 mmol/L). Glucose was not present in the incubation mixtures if cytochalasin B or ConA were being used in the experiments or if HMPS activity was being measured. All assays involving PMN, except the spectrophotometric assays for O_2^- production, were performed in plastic containers.

Stock solutions were made as follows: ConA at 2 mg/mL in distilled water; PMA at 2 mg/mL in dimethyl sulfoxide (DMSO) and diluted to 20 $\mu\text{g}/\text{mL}$ in buffer; cytochalasin B at 1 mg/mL in DMSO; serum-treated zymosan (STZ) at 20 mg/mL in buffer after the treatment of zymosan with serum at 20 mg/mL for 30 minutes at

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37 °C,¹⁶ and FMLP at 1 mmol/L in DMSO and diluted to various concentrations in buffer as needed.

Incubation of PMN With BCNU

PMN at a concentration of 1 to 2×10^7 /mL in PBS with glucose were incubated for ten minutes at 37 °C with varying concentrations of BCNU in DMSO or DMSO such that the final concentration of DMSO was always less than or equal to 0.5% of the total volume. After the ten-minute incubation, 5 vol of ice-cold PBS were added and the cells centrifuged at 500 g for ten minutes at 4 °C. The PMN were then washed once with 10 mL PBS and finally resuspended in PBS with or without glucose (as mentioned earlier) to the same cell concentration as initially used for the incubation. When additional incubations in glucose oxidase were performed, the cells were incubated at 37 °C for 20 minutes in PBS with glucose, with or without GO (80 µg/mL). This amount of GO resulted in a flux of H_2O_2 of 1.2 µmol/min/mL. After the incubation, the cells were washed once with PBS and resuspended at the concentrations desired in PBS with or without glucose.

GRED Activity

GRED was quantitated spectrophotometrically as described by Beutler.¹⁷ The assay cuvettes contained 100 µmol/L NADPH, 1 µmol/L FAD, and PMN in the presence of 0.05% Triton X-100, 50 mmol/L Tris, and 100 µmol/L EDTA at pH 8.0. The assay was started by the addition of 3.3 µmol/L GSSG to the sample cuvette only, with the total volume in both reference and sample being 1.0 mL.

HMPS Activity

PMN at a final concentration of 1×10^6 /mL were incubated for 30 minutes (unless otherwise stated in the Results section) at 37 °C in Krebs' Ringer's phosphate (KRP: PBS plus 1 mmol/L $CaCl_2$ and 1.3 mmol/L $MgSO_4$, pH 7.4) containing 1 mmol/L glucose and 0.1 µCi ^{14}C -glucose, either alone or in the presence of 1 mmol/L L-leucine and 0.1 U/mL LAAO, 0.1 mmol/L methylene blue, 1 µg/mL PMA, 4 mg/mL STZ, or varying amounts of t-BuOOH, all in a total volume of 1 mL in septum-stoppered test tubes with a suspended central well containing 0.2 mL hyamine hydroxide.¹⁸ At the end of the incubation, 2.0 mL of 5 mol/L H_2SO_4 was added and the incubation continued for an additional 30 minutes. The amount of LAAO used resulted in a flux of H_2O_2 of 60 nmol/min/mL. The trapped radioactive CO_2 was then quantitated in a liquid scintillation counter.

Nonprotein Reduced Sulphydryl Levels in PMN

PMN (1×10^7 in 1 mL) were rapidly centrifuged in a Fisher microcentrifuge. The resulting cell pellet was treated with 0.2 mL of 1.0% Triton X-100 and 0.3 mL of protein-precipitating solution¹⁹ and centrifuged for five minutes in a microcentrifuge at 4 °C. Reduced sulphydryl levels were obtained on the resulting supernatant using DTNB as described for erythrocytes by Beutler.¹⁹

GSH Levels in PMN

PMN were centrifuged, treated with Triton X-100 and the protein-precipitating solution, and clarified by centrifugation as earlier. The GSH levels were determined as described by Hissin and Hilf,²⁰ in which 0.01- to 0.1-mL samples were added to 0.1 mol/L sodium phosphate, pH 8.0, containing 0.1 mL of 0.1% OPT in absolute methanol in a final volume of 2.0 mL. After 15 minutes' incubation at 25 °C, the fluorescence of the samples was read in a Perkin-Elmer model 650-10S spectrofluorometer with the excitation wavelength set at 350 nm and the emission at 420 nm. GSH

standards were assayed at the same time. The recovery of GSH, determined by adding known amounts of GSH to the samples, was always greater than 90%.

Bipolarity Assay

The ability of PMN to respond to FMLP by assuming a bipolar shape was measured by modifications of a previously described technique.²¹ PMN were washed and resuspended at 5×10^5 /mL in PBS. Flat-bottomed 96-well tissue culture plates were coated with 50 µL of 1% bovine serum albumin (BSA) for 15 minutes at 37 °C. After removing the BSA solution, 50 µL of PMN was added and allowed to settle onto the plates on ice for 30 minutes. The cells were then warmed at 37 °C for five minutes, and FMLP or control solutions were added and incubated with the addition of 200 µL of cold 5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. The supernatant was removed by aspiration after 15 minutes and replaced with fresh cacodylate buffer. The percentage of bipolar cells was counted using an inverted microscope.

Degranulation

STZ (4 mg) was added to 2×10^6 PMN in a final volume of 1.0 mL KRP in the presence of cytochalasin B (5 µg) for 30 minutes at 37 °C. The cells were then placed on ice and centrifuged and the supernatants assayed for the β -glucuronidase²² and lysozyme²³ content. Whole cells were also assayed for their enzyme content. All assays contained 0.05% Triton X-100. The specific degranulation was determined by subtracting the control supernatants from supernatants of zymosan-treated cells. As an index of cell lysis, the lactic dehydrogenase (LDH) release was measured.²⁴

Superoxide Production

Superoxide production by cells incubated as described was performed in a continuous spectrophotometric assay as previously described²⁵⁻²⁷ using either PMA (1 µg), ConA (100 µg), or STZ (4 mg) as the stimulus. In some assays, 0.1 mL cytochrome c was added to both sample and SOD-containing reference cuvettes either two or ten minutes after the addition of the stimulus to measure the rate of O_2 production at that time. PMA-stimulated NBT dye reduction was performed by incubating 2.5×10^6 PMN with 1 µg PMA in KRP buffer containing 0.04% NBT and 1% albumin in a total volume of 1.0 mL at 37 °C for five minutes. The reaction was terminated by the addition of 5 mL ice-cold PBS and the cells centrifuged at 280 g for five minutes. After washing the cells once, the cells were resuspended in 0.02 mL of 5% albumin and smeared on glass slides. The dried slide was fixed in methanol, dried again, and counterstained with 1% safranin for one minute. Two hundred consecutive cells were counted for formazan deposits.

Ingestion

The ingestion of ^{14}C -labeled *Escherichia coli* was measured in cells that had been incubated as described earlier using a modification of the method of Newburger et al.²⁸ ^{14}C -labeled bacteria were prepared by 24-hour growth of *E. coli* in minimum essential medium containing 3 mmol/L glucose, 100 µCi U- ^{14}C -glucose, and 50 µCi U- ^{14}C -L-leucine. Bacteria were washed, autoclaved, and resuspended at approximately 5×10^8 /mL. Prior to the assay, the bacteria were washed with saline, resuspended in 2 mL fresh human serum, opsonized for 30 minutes at 37 °C, and then washed free of serum. PMN (2×10^6) were incubated with opsonized bacteria (approximately 2×10^7) in KRP with and without 1 mmol/L NEM in the presence or absence of 0.5 mmol/L NaCN for 30 minutes at 37 °C. After the incubation 1 mmol/L NEM was added to all samples and the PMN washed three times with PBS followed by

centrifugation at 200 g for five minutes. The final pellets were counted in a liquid scintillation counter. Ingestion was corrected for adherence by subtracting the radioactivity of cells incubated in the presence of NEM from that in its absence. The results are expressed as counts per minute associated with the PMN.

RESULTS

The Effect of BCNU on PMN GRED Activity

Because BCNU has been shown to inhibit the GRED activity of mammalian cells, we determined its effect on this activity in PMN. PMN were incubated for ten minutes in the presence of varying concentrations of BCNU. The cells were then washed twice and assayed for GRED activity in the presence of 1.0 $\mu\text{mol/L}$ FAD (a cofactor for GRED) and 0.05% Triton X-100. We had previously shown that the Triton X-100 treatment of granulocytes results in complete expression of GRED activity but does not inhibit the enzyme activity. The results are presented in Fig 1. As can be seen, there is a concentration-dependent inhibition of PMN GRED by BCNU, with 50% inhibition occurring at approximately 2 $\mu\text{g/mL}$ BCNU. A ten-minute incubation was chosen because it ensured maximum inhibition and did not affect O_2^- production by activated PMN. Because BCNU inhibition of GRED requires a catalytically active enzyme,¹⁴ all inhibition values pertain to the assay of enzymatic activity in cell lysates made after incubation of the cells with BCNU. Incubation of PMN with 10 to 50 $\mu\text{g/mL}$ BCNU resulted in less than a 6% loss of viability when compared with control cells as assessed by either trypan blue exclusion or the retention of LDH.

The Effect of GRED Inhibition by BCNU on HMPS Activity

We determined the effect of BCNU on the ability of PMN to metabolize H_2O_2 through the glutathione cycle and the HMPS. We used L-leucine and L-amino acid oxidase as an exogenous H_2O_2 -generating system. Figure 1 compares the

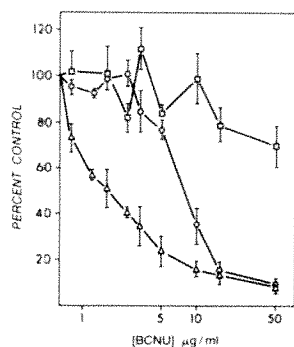


Fig 1. Effects of BCNU on GRED and HMPS activity. PMN that had previously been incubated with DMSO or BCNU at the concentrations noted as described in Materials and Methods were assayed for GRED activity (Δ — Δ) or assayed for HMPS activity using either L-leucine plus LAAO ($\circ$ — $\circ$) or methylene blue ($\square$ — $\square$) as a stimulant. The results are reported as the percentage of activity in DMSO-treated cells. The values represent the means $\pm$ SD for between three and nine samples.

results obtained when comparing the effects of BCNU on both GRED (triangles) and H_2O_2 metabolism through the HMPS (circles). Significant inhibition of the metabolism of H_2O_2 does not occur until the GRED levels decrease to approximately 30% of the control values. Fifty percent inhibition of H_2O_2 metabolism occurs at approximately 8 $\mu\text{g/mL}$ BCNU.

To determine that the effect of BCNU was on the glutathione cycle and not the HMPS itself, we examined the effect of BCNU on the shunt directly. When methylene blue is added to cells, it directly oxidizes NADPH and thereby stimulates the HMPS. Figure 1 also shows the effect of BCNU on methylene blue-stimulated HMPS (squares). Methylene blue-stimulated HMPS activity in BCNU-treated PMN is approximately equal to control PMN at concentrations of BCNU that result in a 70% to 80% inhibition of both H_2O_2 metabolism and GRED activity. This indicates that the ability of the HMPS to function is not affected by BCNU concentrations that inhibit the glutathione cycle.

We also investigated the effect of BCNU on the ability of PMN to reduce known quantities of t-BuOOH. As can be seen in Fig 2, increasing concentrations of BCNU resulted in a progressive inability to reduce t-BuOOH by using the reducing equivalents generated through the HMPS.

For most of the studies to be reported, we tried to determine the effect of the diminished ability to metabolize H_2O_2 on PMN function. We therefore used a concentration of BCNU (10 $\mu\text{g/mL}$) that inhibited H_2O_2 metabolism by 70% but did not, even in the presence of an H_2O_2 -generating system, result in demonstrable cell lysis.

The Effect of GRED Inhibition on a Reduced Sulphydryl Content and Glutathione Content of PMN

Because GRED is involved in maintaining reducing equivalents in cells, we measured the effect of the incubation of PMN and BCNU (10 $\mu\text{g/mL}$) on a reduced sulphydryl

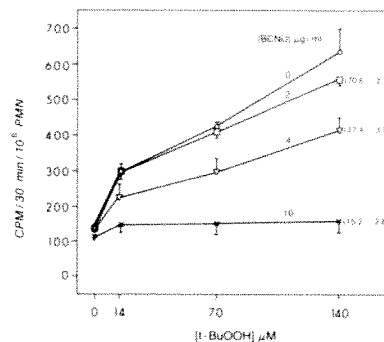


Fig 2. Effect of BCNU on the HMPS activity in response to t-BuOOH. PMN that had previously been incubated with DMSO ($\circ$), 2 μg BCNU ($\square$), 4 μg BCNU (∇), or 10 μg BCNU ($\times$) in 1 mL of PBS were washed and resuspended in PBS. The PMN were assayed for GRED activity, and the HMPS activity was measured in the presence of t-BuOOH at the concentrations noted. The values in parentheses refer to the percentage of control GRED activity found in these PMN after incubation with the aforementioned amounts of BCNU. The points represent the means $\pm$ SD for three determinations.

content. We compared BCNU-treated cells with control cells for their reduced sulfhydryl content and then incubated cells in either buffer or an H_2O_2 -generating system (glucose plus GO) in the presence and absence of cyanide. Reduced sulfhydryl levels were measured with DTNB, and the results are presented in Fig 3. The incubation of PMN with BCNU resulted in a decrease in the reduced sulfhydryl levels. Further incubation at 37 °C for 20 minutes in buffer did not dramatically change this level. After exposure to an H_2O_2 -generating system, the reduced sulfhydryl content of the control PMN did decrease. However, BCNU-treated PMN had a greater fall in their reduced sulfhydryl level than did the control PMN. The addition of cyanide to this system resulted in an accentuation in the decrease in reduced sulfhydryl level in BCNU-treated cells. We also investigated the effect of a phagocytic stimulus on the reduced sulfhydryl content in control and BCNU-treated PMN. The addition of STZ to control cells resulted in a depletion in the reduced sulfhydryl content. This effect was accentuated in BCNU-treated cells (data not shown).

We used the method of Hissin and Hilf²⁰ to determine the effect of BCNU inhibition of GRED on the GSH levels in PMN. Cells were treated in the same way as described earlier; the results are shown in Table 1. After incubation with BCNU, the GSH levels were decreased. With incubation in an exogenous H_2O_2 -generating system, the GSH levels were decreased to a greater extent in the BCNU-treated cells. Thus it appears that in the absence of adequate GRED activity GSH and other reduced sulfhydryls are diminished and are further affected by the addition of an exogenous source of H_2O_2 .

The Effect of GRED Inhibition on Phagocytic Function

FMLP-induced shape changes. It has previously been shown that the inability of PMN to change their shape and become bipolar is a very sensitive indicator of the effect of

Table 1. Effect of BCNU With or Without GO on the GSH Content of PMN

Incubation	GSH (nmol/10 ⁶ PMN)	
	DMSO	BCNU
None	2.90 ± 0.57*	1.95 ± 0.63*
20' PBS + Glu	2.70 ± 0.59†	1.83 ± 0.73‡
20' PBS + Glu + GO	2.49 ± 0.56†§	1.23 ± 0.70‡§

PMN (1×10^7) that had been incubated with DMSO or BCNU (10 μ g/mL) were then either directly assayed for the GSH and GSSG content or incubated for 20 minutes at 37 °C in PBS with 5 mmol/L glucose with or without GO (80 μ g) in a total volume of 1.0 mL. The PMN were then assayed for their GSH and GSSG content. The results are expressed as means ± SD for ten experiments. Statistics (paired *t* test) are presented for the comparisons of conditions as indicated by the superscripts.

**P* = .0011.

†*P* = .14.

‡*P* = .0075.

§*P* = .000013.

the chemoattractant FMLP.²¹ We examined the effect of low (1 μ g/mL) and high (10 μ g/mL) concentrations of BCNU on this aspect of chemotactic responsiveness. This assay was chosen because it examined cells individually, and the effects of both sensitivity to FMLP and the total number of cells responding could be measured. Incubation of PMN with either 1 or 10 μ g/mL BCNU had no major effect on either the sensitivity of the cells to FMLP or the number of cells responding to FMLP, as can be seen in Fig 4. However, if cells treated with 10 μ g/mL BCNU were then treated with glucose plus glucose oxidase, many of the cells lost their ability to respond to FMLP. Of note is that neither the control cells nor cells treated with 1 μ g/mL BCNU were affected by exposure to an exogenous H_2O_2 -generating system. The effect of the high concentration of BCNU was on the number of cells responding to FMLP, not on the relative sensitivity of the cells responding. Thus, BCNU pretreatment can make some PMN more sensitive to the effects of an

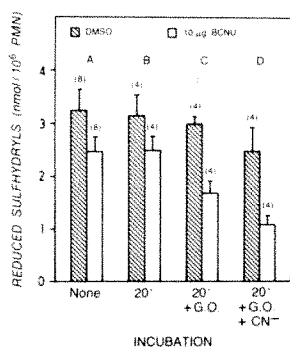


Fig 3. Effect of BCNU on the reduced sulfhydryl content of PMN. PMN that had previously been incubated with DMSO or BCNU (10 μ g/mL) as described in Materials and Methods were assayed for reduced sulfhydryl content (A). Additional PMN (1×10^7) were incubated for an additional 20 minutes in PBS with 5 mmol/L glucose alone (B), with 80 μ g GO (C), or with 80 μ g glucose oxidase and 0.5 μ mol NaCN (D) in a total volume of 1.0 mL at 37°C prior to being assayed for reduced sulfhydryl content. The results are reported as the means ± SD for the number of samples as shown in the parentheses.

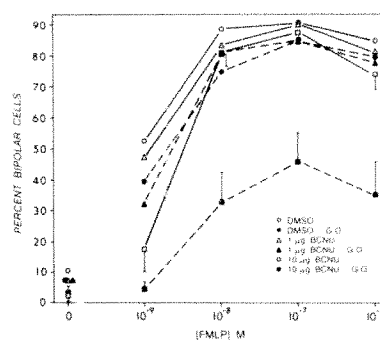


Fig 4. Effect of BCNU with or without GO on FMLP-induced PMN bipolarity. PMN (1×10^7) that had been incubated with DMSO (○,●), 1 μ g/mL BCNU (△,▲), or 10 μ g/mL BCNU (□,■) as described in Materials and Methods were then incubated for 20 minutes in PBS with 5 mmol/L glucose (solid lines) or in the same buffer containing 80 μ g GO (dashed lines) in a total volume of 1.0 mL. The PMN were then washed and assayed for a bipolarity response to the concentrations of FMLP noted on the abscissa. The results are the means of five experiments. SD are given only for the 10- μ g/mL BCNU samples.

H₂O₂-generating system such that they are unable to respond to FMLP with a shape change.

Degranulation. Degranulation was measured by examining the supernatants for the release of β -glucuronidase and lysozyme after stimulation with STZ in the presence of cytochalasin B. The effects of BCNU with and without exposure to an H₂O₂-generating system can be seen in Fig 5. Treatment of PMN with 10 μ g/mL BCNU resulted in a statistically significant ($P < .05$) decreased ability of these cells to degranulate in response to STZ. After the control and BCNU-treated cells were incubated in the presence of glucose plus GO, there was a decrease in their ability to degranulate in response to STZ. The BCNU-treated cells displayed a greater sensitivity to the exogenous H₂O₂-generating system than did the control cells ($P < .0005$). Qualitatively similar results were obtained when lysozyme release in response to STZ was measured (data not shown). These results are similar to those seen for the FMLP shape change and again indicate a sensitivity of BCNU-treated cells to the effects of an H₂O₂-generating system. In addition, studies we were able to determine that the degree of inhibition of the STZ-stimulated release of β -glucuronidase was dependent on the amount of GO used in the incubation when the amount of GO was varied from 1 to 80 μ g/mL. At each concentration, the inhibition was greater for the BCNU-treated cells when compared with the control cells.

Oxidative metabolism. To determine the effect of GRED inhibition on the ability of the cells to undergo an oxidative burst in response to stimuli, we examined the effect of BCNU treatment on O₂⁻ production by PMN stimulated with PMA, ConA, or STZ. Table 2 shows that BCNU treatment alone had no effect on the rate of O₂⁻ production by cells activated by any of the stimuli. Interestingly, even after incubation of the cells with glucose plus GO there was no alteration in the rate of O₂⁻ production with any of the stimuli. However, when cells were incubated in a glucose-plus-GO system in the presence of cyanide (an inhibitor of

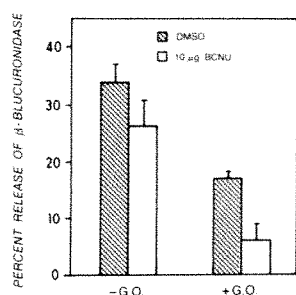


Fig 5. Effect of BCNU on STZ-induced β -glucuronidase release from PMN. PMN (2×10^6) that had been incubated with DMSO or BCNU (10 μ g/mL) and then with PBS plus 5 mmol/L glucose with or without 80 μ g/mL GO and washed as described in Materials and Methods were incubated in KRP with 5 μ g cytochalasin B with or without 4 mg STZ in a total volume of 1.0 mL for 30 minutes at 37°C. The cells were centrifuged, and the amount of β -glucuronidase in the supernatants was determined after correcting for release in the absence of STZ. The results are expressed as the mean percent release $\pm$ SD of β -glucuronidase specifically released for four experiments.

Table 2. Effect of BCNU on the Sensitivity of PMN O₂⁻ Production to H₂O₂

Incubation	Stimulant	Additions (nmol O ₂ ⁻ /min/10 ⁶ PMN)		
		None	GO	GO + NaCN
DMSO	PMA	2.23 $\pm$ 0.68	2.21 $\pm$ 1.32	2.18 $\pm$ 0.93
	ConA	3.03 $\pm$ 0.73	2.75 $\pm$ 0.80	2.57 $\pm$ 1.10
	STZ	2.00 $\pm$ 0.30	ND	1.88 $\pm$ 0.43
BCNU	PMA	2.91 $\pm$ 0.68	2.58 $\pm$ 0.89	1.19 $\pm$ 0.91*
	ConA	3.08 $\pm$ 0.68	2.26 $\pm$ 1.02	0.53 $\pm$ 0.02†
	STZ	1.91 $\pm$ 0.35	ND	0.61 $\pm$ 0.72‡

PMN (2×10^6) that had been incubated with DMSO or BCNU (10 μ g/mL) were then incubated in PBS plus 5 mmol/L glucose alone or with 80 μ g GO with or without 0.5 μ mol NaCN for 20 minutes at 37 °C in a total volume of 1.0 mL. The cells were then washed and assayed for their ability to generate O₂⁻ in the presence of PMA (1 μ g), ConA (100 μ g), or STZ (4 mg) in KRP with 5 μ g cytochalasin B in a total volume of 1.0 mL. The results are the means $\pm$ SD for five experiments. The statistical analysis used the paired *t* test.

Abbreviation: ND, not determined.

* $P < .01$ when compared with no addition.

† $P < .001$ when compared with no addition.

‡ $P < .02$ when compared with no addition.

Other results are not statistically significantly different from no addition values.

catalase), the resulting rate of O₂⁻ production by BCNU-treated cells was diminished when compared with control PMN. Incubation of PMN in cyanide alone had no effect on their rate of O₂⁻ production (data not shown). Thus, although BCNU inhibition of GRED did affect the cells' ability to metabolize H₂O₂, it was not until an alternative method of metabolizing H₂O₂ was inhibited that oxidative metabolism was affected.

We used NBT dye reduction to determine whether the observed decrease in O₂⁻ production was due to an inability of a subpopulation of PMN to respond to a stimulus, as was seen in the bipolarity assay. With PMA as a stimulant, there was only a slight reduction in the number of NBT-positive cells, from 96% in control cells to 90% in BCNU-treated cells, that had been incubated with glucose plus glucose oxidase in the presence of cyanide. Using this assay, we noted that the intensity of the NBT positivity of the cells was markedly diminished in the BCNU-, glucose-, GO- and cyanide-treated cells.

Cytochalasin B was used in the investigations on O₂⁻ production because its presence is necessary for ConA-stimulated O₂⁻ production²⁶ and enhances the detection of O₂⁻ by STZ-stimulated PMN.²⁷ Cytochalasin B has no effect on PMA-stimulated O₂⁻ production. The inhibition of PMA-stimulated O₂⁻ production in BCNU-treated cells incubated in the presence of glucose plus GO with cyanide was also found in the absence of cytochalasin B.

Because PMN can produce their own H₂O₂ through the respiratory burst, we examined the effect of BCNU treatment on the duration of the respiratory burst by comparing the O₂⁻ production at two minutes after stimulation with the O₂⁻ production at ten minutes after stimulation. For this experiment, cytochrome c was not added until either two or

ten minutes because its presence precludes the dismutation of O_2^- to H_2O_2 . As can be seen in Table 3, the O_2^- production of BCNU-treated cells was diminished at ten minutes after stimulation with PMA when compared with the O_2^- production at two minutes. In contrast, there was no difference in the ratio of ten-minute to two-minute points when either STZ or ConA was used as a stimulus. Comparing BCNU-treated cells and control cells for stimulus-dependent HMPS activity as a function of time showed similar results. Figure 6 shows that there is a progressive decrease in the ratio of HMPS activity with time in BCNU-treated cells when compared with control cells when PMA was the stimulant. This effect was much diminished when STZ was used as a stimulant. The HMPS activity could not be assessed for ConA, because ConA inhibited the assay system even when dihydrocytochalasin B was used to enhance ConA stimulation of oxidative metabolism.

Ingestion. We next examined ingestion of opsonized ^{14}C -labeled *E. coli*. Ingestion as distinct from attachment, was measured by comparing the amount of radioactivity associated with the PMN in the presence and absence of 1 mmol/L NEM. The difference was used as an index of the number of *E. coli* ingested. As can be seen in Table 4, BCNU-treated cells displayed the same ability to ingest ^{14}C -labeled *E. coli* as did control cells. In addition, preincubation of the cells in glucose plus GO by using up to four times as much GO as used in the other studies had no effect on the ability of the cells (either control or BCNU-treated) to ingest these particles in either control or BCNU-treated cells, even in the presence of cyanide. Thus, despite the effects of BCNU and H_2O_2 on the GSH and reduced sulfhydryl content, degranulation, ability to respond to a chemoattractant, and oxidative metabolism, there was no effect of BCNU either with or without an H_2O_2 -generating system, even in the presence of cyanide, on the ingestion of ^{14}C -labeled *E. coli*.

Table 3. Effect of BCNU on the Rate of Decrease of PMN O_2^- Production

Stimulant	Ten-Minute Rates of O_2^- Production as a Percentage of the Two-Minute Rate		P
	DMSO	BCNU	
PMA	79.8 ± 17.3	40.6 ± 7.42	<.02
ConA	39.6 ± 17.0	37.2 ± 12.3	NS
STZ	81.1 ± 20.3	86.2 ± 18.3	NS

PMN (2×10^6) that had been incubated with DMSO or BCNU (10 μ g/mL) as described in Materials and Methods were washed and assayed for O_2^- production in KRP containing 5 μ g cytochalasin B and either PMA (1 μ g), ConA (100 μ g), or STZ (4 mg) in a total volume of 1.0 mL. The rates of O_2^- production were determined at two and ten minutes after the addition of the stimulant as described in Materials and Methods. The results are expressed as the means of the percentage of the ten-minute rate compared with the two-minute rate $\pm$ SD for four experiments. The control rates of O_2^- production in the absence and presence of BCNU respectively were 4.19 ± 0.65 and 3.85 ± 0.34 (PMA), 3.27 ± 0.39 and 2.88 ± 0.32 (ConA), and 3.94 ± 0.66 and 3.63 ± 0.83 (STZ) nmol O_2^- /min/ 10^6 PMN. The statistical analysis used the paired *t* test.

Abbreviation: NS, not significant.

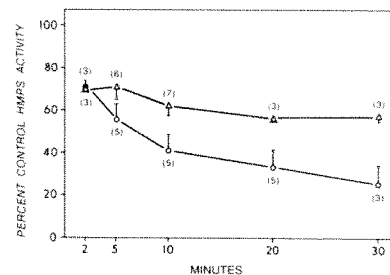


Fig 6. Comparison of PMA and STZ stimulation of HMPS activity in BCNU- and DMSO-treated PMN. PMN (1×10^6) that had been incubated with DMSO or BCNU (10 μ g/mL) and washed were assayed for HMPS activity as described in Materials and Methods in the presence of PMA (1 μ g) (○) or STZ (4 mg) (Δ). At the times noted the reactions were terminated by the addition of 5 mol/L H_2SO_4 , and the $^{14}CO_2$ was measured. The results are expressed as the percentage of counts per minute in the BCNU-treated cells as compared with the DMSO-treated cells at each time point. The number of experiments for each time point are as noted in the parentheses. The upper numbers relate to the STZ experiments and the lower numbers to the PMA experiments.

DISCUSSION

The oxidative burst that accompanies phagocytosis is of short duration.^{10,29} This is probably important because the products of the oxidase system may be toxic to the host. It is believed that H_2O_2 , a product of the burst, is at least partially responsible for limiting its activity. The PMN of patients with complete GRED deficiency demonstrated an abbreviated respiratory burst with a greater and more rapid fall in the reduced sulfhydryl content than normal PMN.¹² Their PMN were unable to undergo an oxidative burst after exposure to an external H_2O_2 -generating system.

To further study the effects of inhibition of GRED on granulocyte function in general, we used the chemotherapeutic agent BCNU, which has been shown to selectively inhibit GRED.^{13,14} As with other cells, we were able to demonstrate a dose-dependent inhibition of cellular GRED by BCNU. The functional consequence of this inhibition, as measured by the ability of the cells to metabolize H_2O_2 , was not found until the GRED activity dropped to between 20% and 30% of control values. This is similar to what we had previously found in rats whose glutathione peroxidase activity was diminished after feeding them a selenium-deficient diet (Baker and Cohen, unpublished observation). Thus, there appears to be a threshold amount of GRED necessary to metabolize H_2O_2 , and this amount is less than that normally present in PMN.

GRED inhibition led to a decrease in the levels of both reduced sulfhydryls and GSH, which may be due to the ability of BCNU-derived alkylating species to directly conjugate with GSH.³⁰ The effect of BCNU on GSH was further accentuated by placing the cells in an H_2O_2 -generating system. This oxidative stress further diminished the GSH content in both control and BCNU-treated cells, but to a greater extent in the BCNU-treated cells. The addition of cyanide to inhibit catalase, which might also be involved in H_2O_2 metabolism, made this effect even more pronounced. The same effect on a reduced sulfhydryl content was seen

Table 4. Effect of GO With or Without NaCN on the Ability of DMSO- or BCNU-Treated PMN to Ingest *E coli*

Incubation	GRED (U/mL/10 ⁷ PMN)	Ingestion (cpm/30 min/2 × 10 ⁶ PMN)		
		Control	GO	GO + NaCN
DMSO	38.4 ± 1.9	10,134 ± 1,163	9,024 ± 1,223	10,908 ± 1,702
BCNU	2.5 ± 0.8	10,528 ± 1,139	10,282 ± 1,418	10,890 ± 1,066

PMN (2 × 10⁶) that had been incubated with DMSO or BCNU (25 µg/mL) were washed and incubated in PBS with 5 mmol/L glucose alone or with 80 µg GO with or without 0.5 µmol NaCN in a total volume of 1.0 mL as described in Materials and Methods. The cells were washed and incubated in KRP containing 5 mmol/L glucose with ¹⁴C-labeled *E coli* that had been opsonized with fresh human serum with or without 1 µmol NEM as described in Materials and Methods. The results are the means ± SD for three experiments. The GRED activity was determined in PMN following incubation in either DMSO or BCNU (25 µg/mL).

when H₂O₂ was generated endogenously by incubating the cells with STZ. Thus, GRED plays an important role in maintaining the levels of PMN reduced sulphydryls and GSH in both the resting and H₂O₂-stressed state.

The effect of the inhibition of GRED and consequent diminution in the reduced sulphydryl content on various aspects of phagocytosis was examined. The results indicate that the various phagocytic functions displayed different sensitivity to GRED inhibition. Prior treatment of cells with BCNU did not affect the responsiveness of cells to a chemoattractant, the ability of cells to undergo oxidative metabolism or to ingest bacteria. The ability of PMN to degranulate in response to a phagocytic stimulus was diminished in BCNU-treated cells. Premature termination of the respiratory burst was found only in BCNU-treated cells that were stimulated with PMA, but not in those stimulated with STZ or ConA. Because stimulation of PMN with PMA occurs by a mechanism different from those associated with other stimulants of the respiratory burst,³¹ this system may be more sensitive to unmetabolized H₂O₂. The amount of GO necessary to produce a sufficient flux of H₂O₂ to produce inhibition of phagocytic function in BCNU-treated cells was much higher than that produced endogenously. This may explain why, with the exception of PMA, there was no premature termination of the respiratory burst and why most other phagocytic functions were unaffected by BCNU alone. The O₂⁻ production by control PMN was lower than that previously reported.²⁵⁻²⁷ We believe that this was due to the multiple incubations and washings required to perform these studies. The stress of an exogenous H₂O₂-generating system on BCNU-treated cells resulted in alterations of some phagocytic functions but not with all functions studied. Thus, responsiveness to a chemoattractant and degranulation

occurred less well in BCNU-treated cells that had been incubated in the presence of glucose plus GO. O₂⁻ production and ingestion were unaffected by this incubation in an exogenous H₂O₂-generating system. However, O₂⁻ production, but not ingestion, was altered when the GO incubation was performed in the presence of cyanide, an inhibitor of catalase. These results indicate that the sensitivity of PMN to H₂O₂ in the presence of decreased GSH is dependent both on the nature of the stimulant and the aspect of phagocytic function.

When comparing the results obtained on O₂⁻ production, ingestion, and degranulation in human PMN treated with BCNU with PMN obtained from glutathione peroxidase-deficient rats,³² it appears that the rat cells are more sensitive to the loss of function of the glutathione cycle. This may be due to the fact that rat PMN are devoid of catalase³³ and thus have no alternative pathway for metabolizing H₂O₂.

It must be noted that, with the exception of degranulation, there was no effect of BCNU alone on any of the phagocytic functions measured. The only instances in which inhibition of phagocytic functions were noted were when the cells were stressed, either endogenously or exogenously, with H₂O₂. Therefore, with the exception of the effect of BCNU on the reduced sulphydryl content, we postulate that the effects of BCNU, as noted, are due to inhibition of GRED and, consequently, H₂O₂ metabolism.

The use of inhibitors of various aspects of the glutathione cycle can be helpful in dissecting the effects of H₂O₂ on components of phagocytosis. However, it is important to realize that these various components display different sensitivity to BCNU and H₂O₂ and that some of the sensitivity may in part be dependent on other protective mechanisms present in PMN.

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Combinations of Recombinant Human Interferons and Retinoic Acid Synergistically Induce Differentiation of the Human Promyelocytic Leukemia Cell Line HL-60

By Hiromichi Hemmi and Theodore R. Breitman

The human acute promyelocytic leukemia cell line HL-60 is induced to differentiate into morphologically and functionally mature monocytelike cells by incubation with a combination of 10 nmol/L retinoic acid (RA) and various concentrations of recombinant immune interferon (rIFN- γ). These induced cells show marked increases in antibody-dependent cellular cytotoxicity (ADCC), antibody-coated erythrocyte (EA) rosettes, nonspecific esterase, and 5'-nucleotidase activity. rIFN- γ alone at concentrations of 10 to 1,000 U/mL has essentially no effect on morphological maturation, nitroblue tetrazolium reduction, and immunophagocytosis. However, rIFN- γ at these concentrations increases EA rosetting in a concentration-dependent manner that is not affected by 10 nmol/L RA. At a concentration of 1,000

U/mL, rIFN- γ induces moderate increases in nonspecific esterase, 5'-nucleotidase, and ADCC. These parameters are markedly increased by the addition of 10 nM RA, a concentration which alone has no effect on these markers. Based on units of antiviral activity, rIFN- γ is tenfold more active than rIFN- α D in inducing EA rosettes and 40-fold more active in inducing nitroblue tetrazolium reduction and immunophagocytosis. These results, indicating that combinations of rIFN- γ or rIFN- α and RA synergistically induce differentiation of HL-60, suggest that this combination may have clinical utility in the treatment of patients with certain leukemias.

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THE HUMAN ACUTE PROMYELOCYTIC leukemia cell line HL-60¹ is a useful model system for studying the differentiation of human myelomonocytic cells.² HL-60 is induced to differentiate into granulocytelike cells by incubation with a wide variety of compounds including dimethyl sulfoxide (DMSO) and retinoic acid (RA)¹⁻³ or into monocyte (macrophage)-like cells by incubation with 1 α ,25-dihydroxyvitamin D₃ and phorbol diesters.⁴⁻⁶ HL-60 is also induced to differentiate into cells having many characteristics of monocyte (macrophage)-like cells by conditioned media of either mitogen-stimulated normal human peripheral blood mononuclear cells or of mixed-lymphocyte cultures.⁶⁻¹² Changes include increases in the percentage of cells positive for nonspecific esterase, having Fc and C3 receptors, capable of immunophagocytosis, reducing nitroblue tetrazolium (NBT), having antibody-dependent cellular cytotoxicity (ADCC), expressing various surface antigens of monocytes or macrophages, and maturing morphologically. Several groups have found that interferon (IFN)- γ , a component of activated lymphocyte-conditioned media, has similar effects on HL-60¹³⁻¹⁸ and the suggestion has been made that this lymphokine is the active component.¹⁴ However, it is apparent from other studies that there are also lymphokines present, distinct from IFN- γ , that have a similar activity.¹⁶⁻¹⁸ We have named these lymphokines "differentiation-inducing activity" (DIA).² A DIA that we have studied was originally described by Olsson and Olofsson¹⁰ and partially purified from normal human stimulated mononuclear cells¹¹ and from the conditioned medium of the malignant T lymphocyte line HUT-102.¹⁹ Although this DIA alone, at a relatively high concentration, induces the differentiation of HL-60, its most remarkable effect is its ability at a relatively low concentration and in combination with the normal plasma concentration of RA (10 to 30 nmol/L) to increase the differentiation of HL-60^{2,19} and fresh cells from patients with acute promyelocytic leukemia.² The findings that IFN- γ appears to have a similar activity prompted the present investigation to study combinations of IFN and RA. A preliminary report on some of the findings in this study has been published.²⁰

MATERIALS AND METHODS

The HL-60 cell line (passages 18 and 28) was maintained in polystyrene tissue culture flasks containing a growth medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). For experiments, the cells were harvested by centrifugation and resuspended in a serum-free defined medium consisting of RPMI 1640 medium supplemented with 5 μ g bovine insulin/mL and 5 μ g human transferrin/mL²¹ at an initial cell density of 2.5 to 3 $\times 10^5$ /mL. The cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air for four days. The cell number was determined with a Coulter Counter, and the viability was determined by trypan blue dye exclusion. RA (all-*trans*- β -retinoic acid, Sigma Chemical Co, St Louis) was dissolved in 100% ethanol and diluted at least 1,000-fold into the growth medium such that the final concentration of ethanol was no higher than 0.1%. Manipulations were in subdued light. Recombinant (r) human IFNs were provided by Dr Sidney Pestka and had the following specific activities (U/mg protein): rIFN- α A, 3 $\times 10^8$; rIFN- α D, 5 $\times 10^7$; and rIFN- γ , 2.6 $\times 10^7$. rIFNs were diluted in RPMI 1640 medium supplemented with 10% FBS. The highest concentration of FBS in the cultures was 0.25%. Measurements of NBT reduction, immunophagocytosis, antibody-coated erythrocyte (EA) rosettes, 5'-nucleotidase (EC 3.1.3.5), and morphology were performed as described in detail.²¹ ADCC against chicken red blood cells (CRBC) was assessed by the method of Perlmann et al.²² CRBC (1 $\times 10^8$) coated with a 1/125 dilution of rabbit IgG against CRBC (Cappel Laboratories, Cochranville, PA) were labeled with 300 μ Ci of ⁵¹Cr (New England Nuclear, Boston) for 1.5 hours at 37°C, washed by centrifugation with phosphate-buffered saline (pH 7.2), and resuspended at a cell concentration of 10⁵/mL in RPMI 1640 containing

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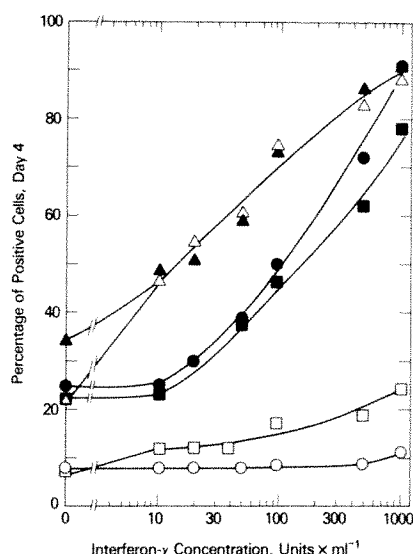


Fig 1. Increases in EA rosettes (Δ , $\blacktriangle$), immunoe erythrophagocytosis ($\square$, $\blacksquare$), and NBT reduction ($\circ$, $\bullet$) of HL-60 cells treated with recombinant IFN- γ alone (open symbols) and in combination with 10 nmol/L RA (closed symbols).

10% FBS. To wells of a 96-well round-bottomed microtiter plate (Linbro Scientific Co, Hamden, CT) 100 μ L of this radiolabeled CRBC suspension (containing 10^4 CRBC/well) was mixed with an equal volume of effector cells suspended in RPMI 1640 containing 10% FBS. Quadruplicates were performed for each condition. The plates were incubated for four hours at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO_2 in air. After incubation the plates were centrifuged, and 100 μ L of the supernatant fraction, containing the ^{51}Cr released from lysed CRBC, was collected and counted in a gamma-radioactive counter. The percent specific cytotoxicity (SC) was calculated according to the formula: Percent SC = $[(S - SR) / TC] \times 100$ where S is the cpm of the sample, SR is the cpm released from target cells (CRBC) alone, and TC is the total cpm in 50 μ L of labeled CRBC. The nonspecific esterase (NSE) was determined with the esterase kit obtained from Sigma Chemical Co by using α -naphthyl acetate as a substrate.

Unless otherwise indicated, data points represent the mean values of at least three determinations in a single experiment. Each

experiment was performed at least two times, and most experiments were repeated at least four times with consistent results.

RESULTS

We studied the effects of rIFN- γ alone and in combination with 10 nmol/L RA on three functional parameters and four other differentiation markers of mature myelomonocytic cells. rIFN- γ alone at concentrations of 10 U/mL or higher increases the percentage of cells forming EA rosettes (Fig 1) and at higher concentrations (1,000 U/mL) increases phagocytosis (Fig 1), the activities of NSE and 5'-nucleotidase, the number of cells with a morphology of monocytelike cells (Table 1), and ADCC (Fig 2) but has essentially no effect on the percentage of cells reducing NBT (Fig 1). The combination of 10 nmol/L RA and rIFN- γ markedly increases the extent of all myelomonocytic differentiation markers compared with the effect of each of these agents alone (Figs 1 and 2 and Table 1). HL-60 treated with 300 nmol/L RA shows increases in both the percentage of cells reducing NBT and maturing morphologically to granulocytelike cells (Table 1) but exhibits essentially no increase in the ADCC or either in 5'-nucleotidase or NSE, two enzyme markers of monocytes/macrophages (Fig 2 and Table 1). rIFN- α D alone, to a concentration of 5,000 U/mL, does not increase the percentage of cells that reduce NBT or are positive for immunophagocytosis. However, a concentration-dependent increase in EA rosetting is observed (Fig 3). At a concentration of 5,000 U/mL of either rIFN- α A or rIFN- α D there is a low percentage of cells expressing NSE activity and maturing to more mature forms (Table 1). Combinations of 10 nmol/L RA and either rIFN- α A or rIFN- α D increase all of these parameters, except for EA rosetting, to values that are greater than what would be expected from an additive effect (Fig 3 and Table 1). Based on U/mL, rIFN- α A and rIFN- α D are much less active than rIFN- γ .

There is no substantial growth inhibition of HL-60 by any of the three rIFNs (Fig 4A and B). There is a maximum growth inhibition of 20% (80% of control) with the rIFN- α s at concentrations of 500 U/mL and higher (Fig 4B), whereas rIFN- γ to a concentration of 3,000 U/mL does not inhibit

Table 1. Morphological and Functional Changes of HL-60 Induced by IFNs and RA

	Myeloid Differential Counts (%)					NBT Reduction (%)	NSE (%)	5'-Nucleotidase Activity (nmol Pi Released/hr/ 10^6 Cells)
	Promyelocyte	Myelocyte	Metamyelocyte	Banded and Segmented Neutrophils	Monocyte			
None	94	4	2	0	0	7	2	0.06
300 nmol/L RA	3	27	32	38	0	97	4	0.04
10 nmol/L RA	73	11	12	4	0	23	1	0.03
rIFN- γ , 1,000 U/mL	86	5	1	0	8	11	12	3.29
10 nmol/L RA plus IFN- γ , 1,000 U/mL	3	12	3	0	82	93	90	12.3
IFN- α A, 5,000 U/mL	84	4	7	1	4	8	11	
10 nmol/L RA plus IFN- α A, 5,000 U/mL	49	8	10	4	29	46	45	
IFN- α D, 5,000 U/mL	88	5	3	0	4	7	12	
10 nmol/L RA plus IFN- α D, 5,000 U/mL	50	10	7	4	29	43	47	

The cultures were incubated for four days.

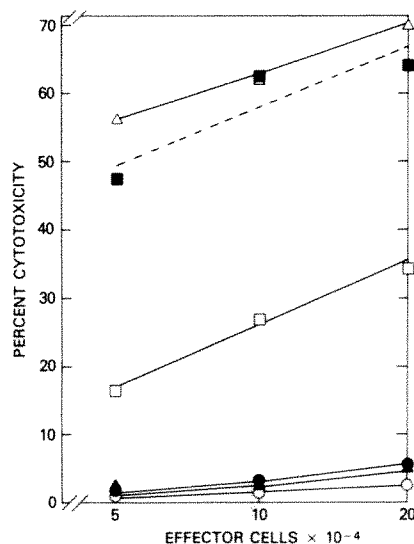


Fig 2. ADCC of HL-60 induced by IFN- γ and RA. HL-60 cells were incubated for four days with none (○), 300 nmol/L RA (●), 10 nmol/L RA (▲), 1,000 U IFN- γ /mL (□), and 1,000 U IFN- γ /mL plus 10 nmol/L RA (■). Normal human peripheral blood mononuclear cells (Δ) were used as a positive control. Under the same conditions there was no spontaneous cytotoxicity of the HL-60 cells against either K-562 or Molt-4.

growth (Fig 4A). In the presence of 10 nmol/L RA, 3,000 U/mL of rIFN- γ decreases the growth to 70% of control compared with a value of 83% of control obtained with 10 nmol/L RA alone (Fig 4A). In the presence of 10 nmol/L RA, the growth of HL-60 with concentrations of rIFN- α s at 3,000 U/mL is approximately 60% of the control growth, and this value decreases to 53% of control at a concentration of 100,000 U rIFN- α D/mL. Under all of these conditions the viability was greater than 80%.

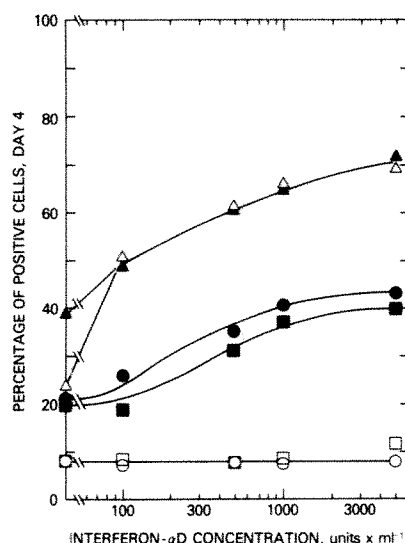


Fig 3. Increases in EA rosettes (Δ, ▲), immunoelectrophagocytosis (□, ■), and NBT reduction (○, ●) of HL-60 cells treated with IFN- α D alone (open symbols) and in combination with 10 nmol/L RA (closed symbols).

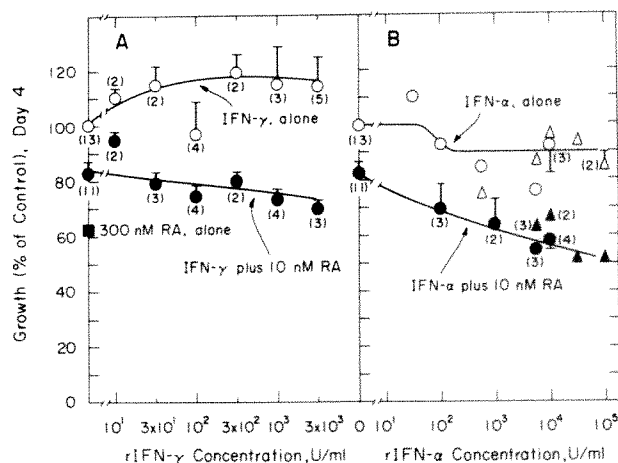


Fig 4. Growth of HL-60 in the presence of IFNs and RA. (A) Growth in the presence of rIFN- γ alone (○) and in combination with 10 nmol/L RA (●). (B) Growth in the presence of rIFN- α A (○, ●) or rIFN- α D (Δ, ▲) alone (open symbols) and in combination with 10 nmol/L RA (closed symbols). The values in parentheses are the number of independent experiments. The bars are the SD. The points with no bars are from one experiment. The mean initial cell concentration was $3 \pm 0.34 \times 10^5$ cells/mL. The mean control cell concentration at day 4 was $10.7 \pm 2.1 \times 10^5$ cells/mL.

DISCUSSION

The major finding in this study is that a combination of RA with either IFN- γ , IFN- α A, or IFN- α D is synergistic for inducing monocytic differentiation of HL-60. There have been many reports on the effects of the IFNs on HL-60 and other human myelomonocytic cell lines. To our knowledge this is the first report to describe the synergistic effects on HL-60 of IFN- γ in combination with RA.

IFN- γ alone increases the concentration of several surface markers including Fc receptors,^{13,14,18,23,24} HLA-DR antigens,^{25,26} chemotactic peptide receptors,¹⁸ and various monocyte-related antigens defined by monoclonal antibodies.^{14,18,24} The increase in the concentration of Fc receptors on HL-60, measured either directly as the number of IgG1-binding sites per cell or indirectly as the percentage of cells forming EA rosettes, is a relatively rapid response to IFN- γ with a maximum increase within 18 hours^{14,23} and with an ED₅₀ (concentration required for a half-maximal effect) of between 6 U/mL¹³ and approximately 40 U/mL.²³ With a four-day incubation period, we find that the ED₅₀ is 40 U/mL (Fig 1). This dose response is not affected by the addition of 10 nmol/L RA even though RA alone at this concentration gives a small increase in Fc-positive cells (Fig 1) and gives values >50% at higher concentrations.² In contrast to other studies^{13,23,24} we find that exposure to IFN- α D results in large increases in the percentage of cells that form EA rosettes (Fig 3). Although Guyre et al¹³ report a 1.6-fold increase in the concentration of Fc receptors on HL-60 after less than a one-day exposure, this increase was observed only at a very high concentration of 10,000 U rIFN- α /mL. Our longer exposure condition is not necessarily an explanation of these differences because it has been demonstrated that even after five days' exposure to 1,000 U rIFN- α /mL there is essentially no increase in Fc recep-

tors.^{23,24} There is no obvious explanation for these differences in results on IFN- α induction of Fc receptors except for the possibility that serum, used in the other studies and not in ours, inhibits the IFN- α induction of Fc receptors. The induction of Fc receptors by rIFN- α A and - α D has been a reproducible finding in our laboratories for over 3 years. In addition, there are increases in two Fc receptor-dependent functions, immunoerythrophagocytosis (Fig 3) and ADCC,²⁷ when HL-60 is exposed to rIFN α in the presence of 10 nmol/L RA. These differences in findings on IFN- α -associated changes in surface antigens are not limited to Fc receptors because IFN- γ is reported either to markedly increase the expression of HLA-DR^{25,26} or to have no effect.²⁴ An increase in the DR antigen is not observed for IFN- α and - β ²⁴⁻²⁶ or for RA, DMSO, or 12-O-tetradecanoylphorbol-13-acetate (TPA).^{12,25} An increase is observed with 1,25-dihydroxyvitamin D₃.²⁵

NSE and the plasma membrane-associated 5'-nucleotidase are two enzyme markers generally accepted to characterize the monocyte/macrophage.²⁸ The 5'-nucleotidase activity increases 55-fold in cells exposed for four days to 1,000 U IFN- γ /mL, and the combination of IFN- γ and 10 nmol/L RA results in an additional fourfold increase in this activity (Table 1). RA alone, either at 10 nmol/L or at 300 nmol/L, does not increase the 5'-nucleotidase activity. Our results with NSE activity (Table 1) are in agreement with Koeffler et al²⁵ and Dayton et al²⁴ who found increases in NSE-positive cells after five to six days of exposure to 1,000 U IFN- γ /mL. A concentration of 100 U/mL is without effect.^{25,29} Thus, although IFN- γ alone, at concentrations of 100 U/mL or less and at relatively short exposure times, induces increases in Fc receptors and HLA-DR, other mature markers of monocytic cells, eg, NSE, do not increase even after four to six days of exposure. At higher concentrations of IFN- γ (1,000 U/mL and higher) there are increases in 5'-nucleotidase and NSE. Exposure of the cells to a combination of 10 nmol/L RA and 1,000 U IFN- γ /mL increases markedly the levels of NSE and 5'-nucleotidase compared with the activities with each agent alone (Table 1). Two other studies report increases in the percentage of NSE-positive cells induced by IFN- γ .^{14,16} In contrast to two other studies^{24,25} we find that IFN- α alone induces a moderate increase in the percentage of cells that are NSE-positive (Table 1). As was the case with IFN- γ , this parameter is increased further by combinations of RA and IFN- α .

Our results on an increase in ADCC by IFN- γ (Fig 2) are in agreement qualitatively with those of Ball et al¹⁴ and Dayton et al.²⁴ ADCC is an activity associated with granulocytes and monocytes/macrophages. It therefore is of interest that HL-60 induced with RA alone at concentrations up to 300 nmol/L has essentially no ADCC activity (Fig 2). A similar finding was reported by Dayton et al¹² for HL-60 induced with 100 nmol/L RA for five days. Because RA induces HL-60 to granulocytelike cells, these results could indicate that the HL-60 granulocyte is deficient in ADCC. In support of this possibility are the findings that DMSO fails to induce ADCC in HL-60.^{14,30} However, it has also been reported that DMSO induces HL-60 to cells with moderate levels of ADCC activity¹² and that monocytoid

cells induced by TPA have essentially no ADCC activity.^{12,30} The combination of 10 nmol/L RA with either IFN- α A or IFN- α D results in the induction of cells with monocytoid characteristics (Table 1 and Fig 3). However, this combination, even with an IFN- α D concentration of 100,000 U/mL, gives only a very small increase in ADCC.²⁷ These results extend those reported previously that IFN- α at a concentration of 1,000 U/mL does not increase the ADCC activity of HL-60.²⁴

A review of the literature on the effects of IFN on the morphology and growth inhibition of HL-60 indicates major differences. Except for one report³¹ there is unanimity that HL-60 is induced by IFN- γ to differentiate to monocytoid cells.^{14,16,32,33} The extent of this change at concentrations of 1,000 U/mL is relatively low. We classified 8% of the cells as monocytes after a four-day exposure (Table 1), whereas Koeffler et al²⁵ found 17% after a six-day exposure. The percentage of monocytes is greatly increased after incubation of HL-60 with the combination of 10 nmol/L RA and 1,000 U IFN- γ /mL (Table 1). IFN- α alone has essentially no effect on changes in morphology (Table 1), in agreement with other reports.^{24,27,32,34} However, in combination with 10 nmol/L RA an increase in morphological maturation is observed (Table 1). Based only on the morphological changes that we saw in the presence of RA and IFN- α it appears that monocytes and more mature granulocytes are formed. However, based on the percent NSE, which is a more objective test for monocytes, it is probable that most of the intermediate granulocyte forms are monocytoid. It is likely that a similar difficulty in distinguishing between intermediate granulocytes and monocytoid cells by light microscopy was also the case with combinations of 10 nmol/L RA and concentrations of IFN- γ <1,000 U/mL.

There have been equivocal results on the inhibition of the growth of HL-60 by the IFNs. Our results (Fig 4A) indicate that IFN- γ alone does not inhibit the growth measured after a four-day incubation period. These results are in agreement with several studies in which the growth or proliferative ability was measured three to six days after the addition of a single dose of IFN- γ .^{14,16,24,29,35} In contrast to these results, four groups report a marked inhibition of the growth of HL-60 by IFN- γ .^{18,31,33,36} In each of these studies natural (n)IFN- γ was used, the incubation period was longer than four days, and/or fresh IFN- γ was added to the cultures during the test period. The greater stability of nIFN- γ compared to rIFN- γ under culture conditions is probably an explanation for these differences.³⁶

The relatively large inhibitory effects on the growth of HL-60 of approximately 50% at concentrations of 500 to 5,000 U IFN- α /mL reported by two groups^{31,32} has not been observed by us (Fig 4B) or by others.^{14,24,34,36} In the presence of 10 nmol/L RA, IFN- α had only a relatively small growth-inhibitory effect (Fig 4A). At a concentration of 3,000 U IFN- α /mL, the growth was 85% of that observed with 10 nmol/L alone. This apparent high growth potential in a population of cells that have many markers associated with terminally differentiated myeloid cells (Table 1, Figs 1 and 2) indicates that the loss of growth potential is a late event in the maturation process. The growth inhibition by

IFN- α in the presence of 10 nmol/L RA (Fig 4B) appears to be greater than that of IFN- γ , especially if comparisons are made at IFN concentrations that induce similar levels of maturation. Thus, it is possible that a portion of the growth inhibition by IFN- α is not related to terminal differentiation.

Our results on the effects of a combination of 10 nmol/L RA and IFN- α confirm and extend those reported by Grant et al.³⁷ These workers found that a combination of 100 nmol/L RA and 1,000 U IFN- α /mL increased by twofold the percentage of cells reducing NBT, from 42% with 100 nmol/L RA alone to 89% with the combination. IFN- α alone did not increase this parameter over the seven-day incubation period. Our results with 10 nmol/L RA in combination with various concentrations of IFN- α also show the apparent synergistic effect not only on NBT reduction but also on phagocytosis, morphology, NSE (Fig 3, Table 1), and more recently on ADCC.²⁷ The marked increase in NSE expression indicates that under our conditions the mature cells are monocytes. No designation of lineage is given by Grant et al.^{32,37} In another study using combinations of RA and IFN- α Tomida et al.³⁴ found after a four-day incubation period that a combination of 40 nmol/L RA and 1,000 U IFN- α /mL had essentially no effect on increasing the percentage of cells maturing beyond the promyelocyte stage compared with the condition with 40 nmol/L RA alone. However, they did find a shift to more mature granulocytes based only on light microscopy evaluation. Because no additional assays were performed to confirm these morphological changes, a shift in the direction of differentiation, from granulocytes in the presence of RA alone to monocytes in the presence of RA and IFN, may have occurred. Under our conditions of 10 nmol/L RA and either IFN- α or IFN- γ there is a shift to the monocyte lineage. It will be of interest to explore in greater depth the question to which lineage HL-60 matures in the presence of various concentrations of RA and IFN.

Recent reports on the binding parameters of the IFNs to lymphocytic and myeloid cells, including HL-60,^{35,38-42} allows us to make some correlations of the biologic effects we and others have observed with the kinetic parameters for IFN binding and the concentration of receptors on the cell plasma membrane. The dissociation constant (K_D) of rIFN- γ for its receptor is approximately 0.4 nmol/L, with approximately 4,000 receptors/HL-60 cell.^{38,40} Based on the specific activity of the rIFN- γ used in this study and a molecular weight of 17,500, a concentration of 0.4 nmol/L is equivalent to 180 U/mL. This concentration of IFN- γ alone increases the Fc receptor concentration of HL-60 and in the presence of 10 nmol/L RA increases the percentage of cells

reducing NBT and capable of phagocytosis (Fig 1). Thus, biologic effects are observed at a concentration of IFN- γ at which 50% of its receptors are occupied and the extent of these effects is closely related to the expected level of receptor occupancy. A similar conclusion can also be made for IFN- α based on a K_D of 0.25 nmol/L.^{42,43} In addition, during RA- or DMSO-induced differentiation of HL-60, the number of IFN- α -binding sites per cell increases almost threefold from 600 to 1,600.⁴³ Because of the expected positive relationship between the receptor number and the extent of a response, cells with a greater number of receptors would exhibit dose-response curves for IFN- α that are shifted to lower concentrations. This in part, may be an explanation for the synergy of combinations of RA and IFN- α . A similar increase in the concentration of IFN- γ receptors has not been observed during differentiation induced with either RA,⁴³ DMSO,^{40,43} or TPA.⁴⁰

Acute myelogenous leukemia has been viewed as a disease involving a block in the normal maturation of myeloid cells. Thus, it is possible that some leukemia cells do not mature either because they have a decreased ability to respond to exogenous differentiative factors or because the production of specific gene products obligatory for differentiation is altered. It is now clear, at least for some human myelomonocytic cell lines, that this block in maturation is not permanent and that incubation with physiological agents promotes end-terminal differentiation. These findings have generated interest in the induction of differentiation as an alternative approach to the treatment of some leukemias. Previous studies have shown that the differentiation of HL-60 in the presence of 10 nmol/L RA is markedly potentiated by agents, including cholera toxin, dibutyryl cyclic adenosine monophosphate (cAMP), and prostaglandin E, that increase the intracellular concentration of cAMP.² In addition, HL-60 is primed to respond to these agents, including the lymphokine DIA, by incubation with 10 nmol/L RA alone.⁴⁴ Fresh cells from patients with acute promyelocytic leukemia are induced to differentiate in primary culture by RA,⁴⁵ and combinations of RA and DIA are even more effective.² Thus, the finding in this and other studies that rIFNs have DIA means that the availability of DIA is not a problem for clinical studies. Recombinant technology should also allow for the construction of new DIAs having greater specificities and/or less toxic side effects.

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Differentiation of Blast Cells From a Down's Syndrome Patient With Transient Myeloproliferative Disorder

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A male neonate with Down's syndrome and congenital myeloproliferative disorder was studied. His blood picture showed the unique coexistence of leukocytosis with matured cells and a large number of blast cells. The *in vitro* proliferation and differentiation of blast cells into various lineages in the presence of phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM) was examined by using a liquid culture and a methylcellulose culture system. The differentiation of blast cells into myeloid cells was confirmed by specific cytochemical stainings, electron microscopy, and an immunologic study. No specific factors in the plasma of the patient promoted the proliferation or differentiation of blast cells. The cellular composition of colonies grown in methylcellulose culture from single blast cells was studied by a micromanipulation technique. High

plating efficiency was observed. Of 136 cultures, 78 showed colony growth. Half of the blast cells were colony-forming cells that could proliferate and differentiate into basophils, neutrophils, eosinophils, macrophages, and erythrocytes in the presence of PHA-LCM. Using the blast cells with a high differentiation capacity to the basophil pathway, we studied the effect of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). Recombinant GM-CSF support neutrophils, eosinophils, and macrophages but not typical basophils. These findings of the cell differentiation of blast cells into various kinds of cells *in vitro* were in agreement with the finding of neutrophilia, eosinophilia, basophilia, and thrombocythemia in this patient.

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INFANTS WITH DOWN'S SYNDROME are predisposed to the myeloproliferative disorder.¹⁻⁶ This myeloproliferative disorder is characterized as follows: (1) the disorder is usually transient and putatively benign; (2) it is not associated with anemia, neutropenia, or thrombocytopenia, despite the presence of many blast cells in the peripheral blood; (3) the percentage of blast cells is greater in the blood than in the bone marrow; and (4) the blast cells demonstrate trisomy 21 with no other cytogenetic abnormalities. The nature of this disorder has been the subject of continued controversy.^{7,8} The etiologic relationship between the leukemoid reaction and acute leukemia is obscure. Because blast cells have no signs of differentiation, the lineage to which these cells belong has not been defined. The capacity of proliferation and differentiation of blast cells has not been clarified yet.

Recently, we encountered a patient with Down's syndrome who showed marked neutrophilia, basophilia, and thrombocythemia with increased numbers of blast cells. We characterized the pattern of proliferation and differentiation of blast cells using the single-cell culture method. Cytochemical, ultrastructural, immunologic, and gene rearrangement studies were done.

CASE REPORT

A 9-day-old boy had typical findings of Down's syndrome with marked hepatosplenomegaly and heart murmur. His hemoglobin level was 18.8 g/dL, and his WBC count was 61,600/ μ L with 62% blasts, 1% promyelocytes, 1% myelocytes, 1% metamyelocytes, 18% segmented neutrophils, 6% eosinophils, 4% basophils, and 7% lymphocytes. Erythroblasts were seen in the peripheral blood. The platelet count was 354,000/ μ L. The bone marrow examination showed a normal maturation of all cell lines despite the presence of 33% blasts. The neutrophil alkaline phosphatase score was within normal limits. A myeloproliferative disorder was diagnosed, and no specific therapy was given. The peripheral WBC increased to 144,000/ μ L with 67% blasts, 10% segmented neutrophils, 10% eosinophils, 3% basophils, and others within 1 month. Platelets also increased to 1,190,000/ μ L. At that time, bone marrow examination showed normal maturation of all cell lines with blasts. The patient gradually became ill because of congestive heart failure. At the age of 2 months, he died of bleeding from a duodenal ulcer. His WBC count was 31,700/ μ L with 11% blasts, 14% segmented neutrophils, 30% eosinophils, 2% basophils, and others one day before death. Autopsy findings showed marked extramedullary hemopoiesis of all cell lines in the liver.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells were separated by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density centrifugation; the interface cells were harvested and washed twice in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY).

Cell culture. Liquid culture was carried out as follows: Cells were suspended in IMDM with 5% phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM) and 10% fetal calf serum (FCS) or the patient's platelet-poor plasma (PPP) or a healthy donor's PPP. The cells were incubated at 1×10^5 /mL in 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY) or in 96-well tissue culture plates (Sumitomo Bakelite Co, Tokyo) at 37 °C in a humidified atmosphere of 5% CO₂ in air. Half of the culture medium was replaced once or twice a week with freshly prepared medium.

Methylcellulose culture was performed in 35-mm nontissue culture dishes (Falcon Labware, Oxnard, CA). A 1-mL mixture of 10^3 peripheral blood mononuclear cells, IMDM, 1.2% methylcellulose

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(Dow Chemical Co, Midland, MI), 30% PPP, 1% bovine serum albumin (Sigma Chemical Co, St Louis), 1 unit of erythropoietin (Toyobo, Osaka, Japan), and 5% PHA-LCM or 5% GM-CSF was incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. PHA-LCM was prepared as described previously.⁹ Recombinant GM-CSF was generously provided by Dr K. Arai in DNAX.¹⁰ Using an inverted microscope, the culture plates were evaluated after 10 days for GM colonies, erythroid bursts, and mixed colonies.

Culture of single cells. The technique for single-cell manipulation was basically similar to that described previously.¹¹ Mononuclear cells were plated on methylcellulose medium at a concentration of 10³ cells/mL in a 35-mm culture dish. The transfer of a single cell to a second 35-mm culture dish containing 1 mL methylcellulose medium with 30% PPP, 5% PHA-LCM, and 1 U/mL of erythropoietin was accomplished under direct microscopic visualization by using a fine Pasteur pipette attached to a micromanipulator (Narishige Scientific Instrument Laboratory, Tokyo). After confirmation that a cell was present in each culture dish, incubation was carried out at 37 °C in an atmosphere of 5% CO₂ in air. On days 11 through 13 of incubation when the colonies appeared to have matured, individual colonies were lifted from the methylcellulose medium using a 3 µL Eppendorf pipette. The samples were spun in a Shandon Cytospin (Shandon Southern Products, Sewickley, PA) and stained with May-Grünwald-Giemsa.

Cytochemical studies. The cells were stained with peroxidase, Sudan black, alkaline phosphatase, toluidine blue, and periodic acid-Schiff (PAS) staining. Nonspecific esterase and chloroacetate esterase staining was performed according to Yam et al.¹²

Immunological studies. Reactivity of the fresh peripheral mononuclear cells and the cultures cells with murine monoclonal antibodies (My 4, 7, 8, Mo 1, Ia and Leu 2, 3, 4, 11, 12, J5) was assayed by an indirect immunofluorescence technique using a fluorescence-activated cell sorter.

Gene rearrangement studies. The status of the immunoglobulin heavy-chain genes and T cell receptor genes was assessed. DNA from blast cells was extracted, digested with restriction enzymes, size fractionated on agarose gels, Southern blotted, and hybridized with the human J_H immunoglobulin gene probe and the human T_β receptor gene probe.

Electron microscopy. Buffy coat cells of peripheral blood and cultured cells were fixed with glutaraldehyde and osmium tetroxide, dehydrated, and embedded in epoxy resin for transmission electron microscopy.

Chromosome analysis. Chromosomes were studied on samples of fresh peripheral blood cells with or without the stimulation of PHA and single colonies according to a method described previously.¹³

RESULTS

Liquid culture. Mononuclear cells isolated from the peripheral blood by Ficoll-Paque density centrifugation showed 86% blast cells. The blast cells had no granules and an undifferentiated appearance on light and electron microscopic examination (Fig 1). Mononuclear cells were cultured in the presence of 5% PHA-LCM and 1 U/mL erythropoietin with 10% PPP or 10% FCS. The total number of cells increased to 100-fold within ten days (Fig 2). The patient's PPP or healthy donor's PPP supported cell growth better than FCS. There was no significant difference between the patient's PPP and healthy donor's PPP. The blast cells had no characteristic cytochemical findings. All surface markers tested were negative. The immunoglobulin heavy-chain

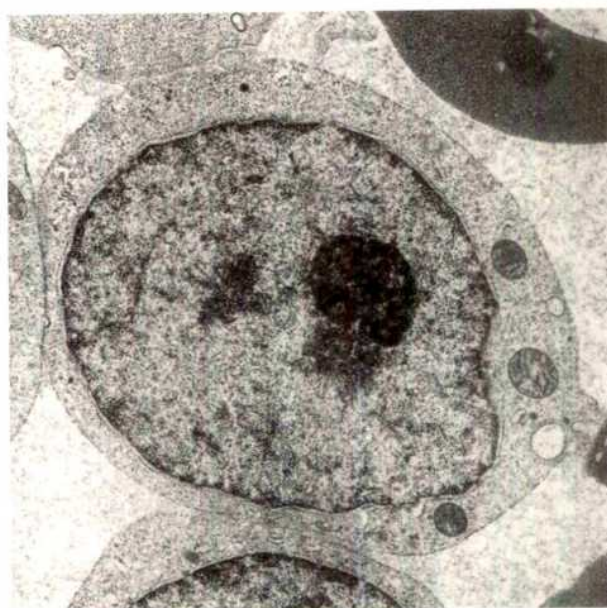


Fig 1. Ultrastructure of a blast cell in the peripheral blood. The round nucleus has finely dispersed chromatin and a large nucleolus; the cytoplasmic organelles are poorly developed (original magnification $\times 10,800$; current magnification $\times 7,560$).

genes and T cell receptor genes of the blast cells were found to be in germ line configuration. However, the cultured cells were positive in peroxidase, Sudan black, alkaline phosphatase, PAS, nonspecific esterase, chloroacetate esterase, and toluidine blue stainings. The cultured cells were reactive with My 7 (antibody-defining antigens expressed on cells in the myeloid lineage) but were not reactive with antibodies of My 4, Mo 1 or Ia. Smears of the cells obtained in a 9-day culture stained with May-Grünwald-Giemsa showed neutrophils, eosinophils, basophils, macrophages, erythrocytes, and megakaryocytes. Electron microscopic studies confirmed these cell lineages.

Methylcellulose culture. We observed a 50% plating efficiency in cultures of patient's peripheral blood mononuclear cells in the medium containing healthy donor's PPP in the presence of PHA-LCM and erythropoietin. The plating efficiency was extremely high when compared with that in cultures of cord blood mononuclear cells (Table 1). Many GM colonies, erythroid bursts, and mixed hemopoietic colonies were observed on the tenth day of culture. The GM

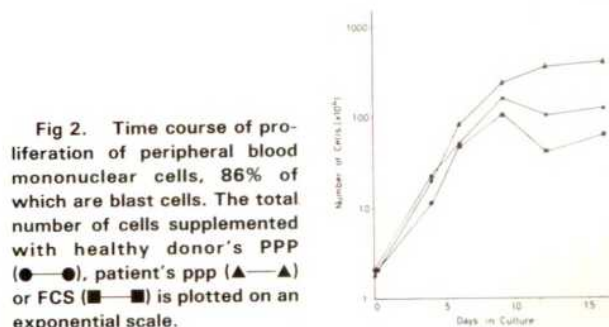


Fig 2. Time course of proliferation of peripheral blood mononuclear cells, 86% of which are blast cells. The total number of cells supplemented with healthy donor's PPP (●—●), patient's ppp (▲—▲) or FCS (■—■) is plotted on an exponential scale.

Table 1. In Vitro Methylcellulose Culture of Mononuclear Cells From the Peripheral Blood of the Patient With Myeloproliferative Disorder and Normal Cord Blood

	Media*	GM Colonies	Erythroid Bursts	Mixed Colonies
Patient	FCS	125 ± 10	17 ± 3	0
	PPP	455 ± 35	10 ± 1	5 ± 5
Cord blood	FCS	6 ± 2	5 ± 1	1 ± 1
	PPP	5 ± 1	3 ± 1	0

Peripheral mononuclear cells were plated at a concentration of 1×10^3 per dish in the presence of 5% PHA-LCM. The colonies were counted on day 10 of culture.

*Media contain 30% FCS or 30% healthy donor's PPP.

colonies consisted mainly of neutrophils, eosinophils, and basophils. Recombinant GM-CSF supported neutrophils, eosinophils, and macrophages but not typical basophils. We examined the cell lineage of constituent cells in colonies grown in a dish containing PHA-LCM or recombinant GM-CSF. About 50% of the cells grown in recombinant GM-CSF contained large granules that were toluidine blue-negative, whereas 50% of the cells grown in PHA-LCM were basophils that were toluidine blue-positive (Table 2). Electron microscopically, the typical basophils grown in PHA-LCM were polymorphonuclear and displayed membrane-bound cytoplasmic granules filled with fine particles (Fig 3A). These ultrastructural features were identical to those observed in human peripheral blood basophils. On the other hand, the cells grown in recombinant GM-CSF lacked the granules with a fine particulated structure but contained large granules that resembled those in young basophils grown in cord blood cell cultures (Fig 3B).¹⁴ We studied

Table 2. Differential Counts of Pooled Colonies in a Dish Containing PHA-LCM or Recombinant GM-CSF

	Differential Counts (%)				Undefined Cells*
	n	m	e	b	
PHA-LCM	52.0	2.0	6.8	39.2	—
Recombinant GM-CSF	44.8	1.6	4.0	—	49.6

Mononuclear cells (1×10^3 /mL) were cultured in the presence of 5% PHA-LCM or 5% recombinant GM-CSF. Differential counting was carried out on 250 cells from ten days of culture.

Abbreviations: n, neutrophils; m, macrophages; e, eosinophils; b, basophils.

*Cells containing large granules that were negative for toluidine blue staining.

colony formation without PHA-LCM, and no colony formation was seen at a concentration of 10^3 cells per dish.

Single-cell culture. To establish the clonality of the colony, we cultured single cells that were transferred using a micromanipulator. Single-cell culture was done in a total of 136 cultures, of which 78 showed colony growth (57%). A total of 66 cultures gave evaluable data. May-Grünwald-Giemsa staining of the colonies revealed various types of colonies as shown in Table 3. Among them, 54 colonies (81.8%) contained basophils, 40 (60.6%) contained neutrophils, 40 (60.6%) contained eosinophils, and 25 (37.9%) contained macrophages. Three colonies contained erythrocytes. Twenty colonies consisted of less than 50 cells. The remaining 47 colonies were larger, and 34 colonies consisted of 200 to 3,500 cells. The appearance of a mixed colony consisting of five lineages derived from a single blast is shown in Fig 4A. The colony showed a compact red center and small

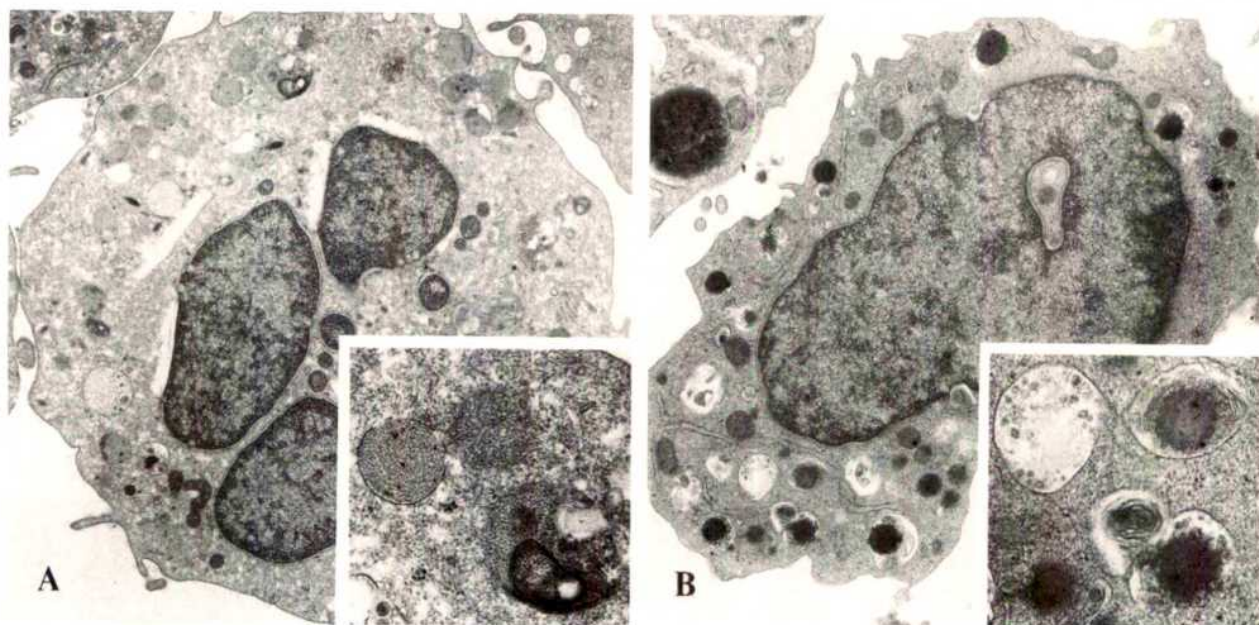


Fig 3. (A) Ultrastructure of mature basophil cultured for nine days in the presence of PHA-LCM. The nucleus is multilobulated. The granules exhibit a fine particulated content, which is typical of basophils in vivo (original magnification $\times 11,200$; current magnification $\times 8,176$). A detailed structure of the granules is highlighted in the inset (original magnification $\times 32,600$; current magnification $\times 23,798$). (B) Ultrastructure of toluidine blue-negative cell grown in the presence of recombinant GM-CSF. The dispersed chromatin of the nucleus indicates the cell is immature. The large granules with homogenous materials in the center are similar to those of basophil/mast cells grown in the culture of cord blood cells (original magnification $\times 11,200$; current magnification $\times 8,176$). A detailed structure of the granules is highlighted in the inset (original magnification $\times 35,700$; current magnification $\times 26,061$).

Table 3. Various Types of Colonies Formed by Single Blast Cells

Lineage					Number of Colonies
b	e	n	m	E	
+	+	+	+	+	2
+	+	+	+		10
+	+	+			15
+	+		+		2
+		+	+		1
+	+				2
+		+			3
+			+		2
+					17
	+	+	+	+	1
	+	+	+		4
	+	+			2
	+		+		1
	+				1
		+	+		1
		+			1
			+		1
54	40	40	25	3	66

The colonies were examined on days 11 through 13 of incubation when the colonies appeared to have matured.

Abbreviation: b, basophils; e, eosinophils; n, neutrophils; m, macrophages; E, erythrocytes.

refractile cells in the surrounding region. Figures 4B and 4C show that the cells consisted of the mixed colony stained with May-Grünwald-Giemsa.

Chromosome analysis. The peripheral blood lymphocytes showed trisomy 21. The sample from peripheral blood without PHA stimulation showed trisomy 21 in all 28 metaphases examined. Of 13 colonies examined, eight colonies contained analyzable metaphases. Each colony contained 1 to 19 metaphases, yielding 55, and all of them were trisomy 21.

DISCUSSION

The hematologic state in the congenital myeloproliferative disorder of Down's syndrome is characterized by increased blast cells and resembles acute congenital leukemia. However, this disorder is unique in that the hematologic abnormality is self-limited. The work of Miller et al¹⁵ and our case show the coexistence of marked thrombocytopenia and a large number of blast cells. This hematologic profile is distinguishable from such myeloproliferative disorders in adults as chronic myelocytic leukemia and essential thrombocytopenia.

An important question in clarifying the characteristics of the blast cells in transient myeloproliferative disorder in Down's syndrome is whether such cells are leukemic or nonleukemic. Leukemia is defined as the clonal proliferation of abnormal cells that are transformed from hemopoietic cells. In our patient, it is difficult to determine whether this disorder is clonal hemopathy or not because there were no additional chromosomal abnormalities or genetic markers. However, it is certain that these blast cells are derived from the cells with trisomy 21 because even in phenotypically normal neonates showing abnormal hemopoietic states, chromosome studies revealed trisomy 21 mosaicism.^{3-6,16} In par-

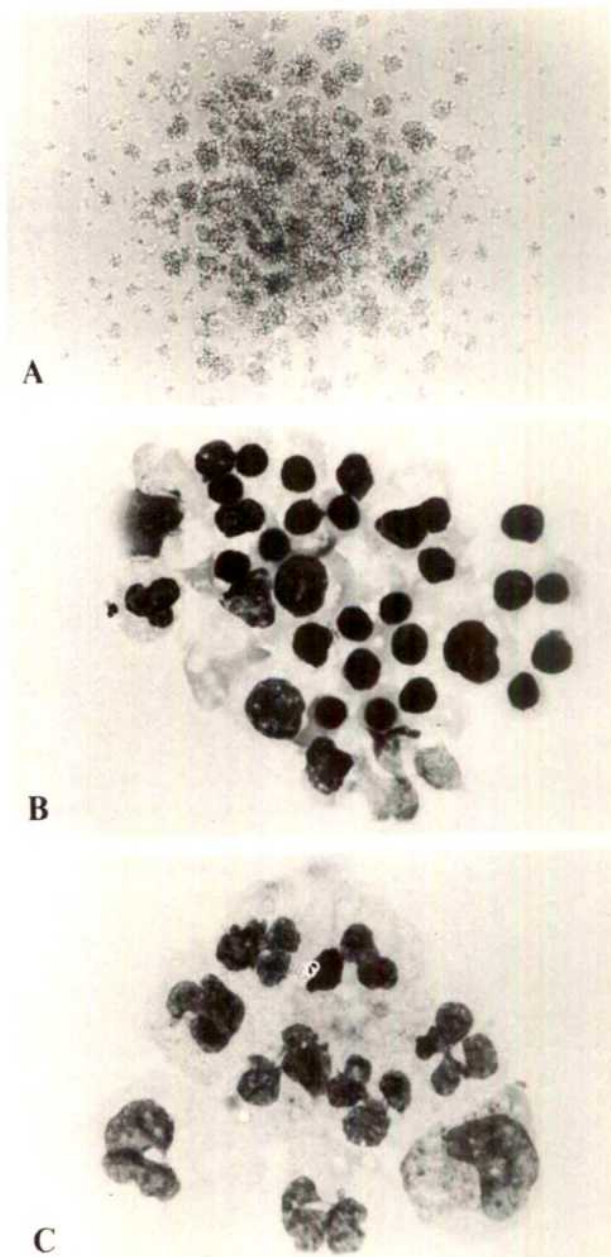


Fig 4. (A) A multilineage colony derived from a single blast cell. The center is compact with small red cells. (B) May-Grünwald-Giemsa preparation of cells from the multilineage colony shown in (A). Erythroblasts are shown. (C) May-Grünwald-Giemsa preparation of cells from the multilineage colony shown in (A). Mature neutrophils and a young myeloid cell are shown.

ticular, Weinberg et al reported a neonate with this disorder having a 46,XX/47,XX,+21 karyotype limited to the hemopoietic system.⁵

The in vitro colony formation was reported to be normal in transient myeloproliferative disorder¹⁷ or transient acute leukemia in Down's syndrome.¹⁸ However, the relationship between blast cells and colony-forming cells is unclear. We demonstrated that blast cells in our patient could proliferate to form large colonies and differentiate into various kinds of cell lineages. The lack of a significant difference between the

patient's plasma and healthy donor's plasma for supporting cell growth suggested that there were no abnormalities of hemopoietic regulators. The single-cell transfer experiment demonstrated that about half of the blast cells were colony-forming cells that could proliferate and differentiate into basophils, neutrophils, eosinophils, macrophages and erythrocytes. Electron microscopy showed the maturation of and differentiation into each lineage.

Nakahata and Ogawa¹⁹ reported a human blast colony assay. However, the replating efficiency of these cells is not as high as those of murine blast colonies.²⁰ The treatment with monoclonal antibody (MY-10) is necessary for the enrichment of stem cells.²¹ The mononuclear fraction of peripheral blood in this patient has an extremely high plating efficiency and differentiation activity. The predominance of colonies containing basophils provided an opportunity for studying whether recombinant GM-CSF supports the differentiation of basophils in addition to neutrophils, eosinophils, and macrophages.¹⁰ It is suggested that recombinant GM-CSF does not support the differentiation of basophils and that other factor(s) might be required for the terminal maturation of basophils.

In acute myelogenous leukemia, the in vitro colony formation was reported to be abnormal; large numbers of small

colonies or clusters were formed, and the constituent cells remained immature.^{22,23} We previously showed that megakaryoblastic leukemic cells also have the capacity for differentiation into some lineages that are not expressed in vivo.⁹ However, in the present case, the findings of the cell differentiation of blast cells into various kinds of cells in vitro were in agreement with the findings of neutrophilia, eosinophilia, basophilia, and thrombocytopenia in vivo.

Before the patient died, granulocytosis developed concurrent with a decrease in the number of blast cells. Autopsy findings revealed extramedullary hemopoiesis in the liver, but the infiltration of blast cells was not marked. In view of the in vitro and in vivo findings, hemopoietic differentiation was not blocked. Therefore, it is assumed that the blast cells of this patient with transient myeloproliferative disorder are transformed from hemopoietic stem cells with trisomy 21 and that these clones could not survive persistently due to their high differentiation activity.

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Relationship of the Hematopoietic Cycle to the Combined Activities of a Stimulator and a Short-Lived Inhibitor

By Philip J. Burke and Judith E. Karp

Substances that circulate in the blood following drug-induced bone marrow aplasia produce a biphasic curve of DNA synthesis in cells in liquid and semisolid cultures which reflect relative concentrations of these growth regulators of hematopoiesis. This net effect magnified by induced marrow failure in human, rat, and mouse is a sinusoidal curve that is the reciprocal of the WBC. Generated in the bone marrow, humoral stimulating activity (HSA) produces peak growth of colonies in agar (CFU-GM) during the proliferative phase of bone marrow recovery, whereas humoral inhibitory activity (HIA), present at the time of marrow maturation, suppresses colony growth. Split femurs from rats given cyclophosphamide (CY) and killed at regular intervals condition media that affect DNA synthesis in a fashion similar to that of HSA-HIA in the rats' sera. In Dexter cultures, HSA is derived from the adherent

rather than the hematopoietic cell, whereas HIA is produced by that nonadherent cell. Animals treated with a lethal dose of busulfan (BU) produce large amounts of HSA in vivo until death. Those transplanted with adherent bone marrow cells depleted of hematopoietic cells on day 1 after BU also die, whereas those given nonadherent marrow cells survive and generate HIA at the time of bone marrow recovery. HSA and media conditioned by bone marrow stromal cells causes an increase in spleen colonies (CFU-S), as does HIA. These studies support the contention that the net effect of putative regulators of hematopoiesis, measured in the drug-perturbed state, consist of a constantly present stimulator emanating from bone marrow stroma, and a variable inhibitor produced by maturing hematopoietic bone marrow cells.

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NORMAL PERIODIC oscillations of the formed elements in the peripheral blood suggest that hematopoiesis is regulated by positive and negative humoral mechanisms.¹ Support for this concept derives from the findings of Bierman² and Morley and colleagues³ of growth-stimulating factors in sera during induced pancytopenia. With an assay capable of measuring the negative phase of a sine-wave, we can detect both stimulatory and inhibitory activities in the sera of patients recovering from drug-induced sublethal bone marrow aplasia.⁴ Sequential studies designed to elucidate this phenomenon yielded data that can now be integrated with emerging evidence for the presence of characterized substances that influence the growth of hematopoietic cells in the bone marrow.⁵

The observed consistent fluctuation of humoral factors during induced acute failure and subsequent recovery of hematopoiesis is best explained by the net activity of a stimulator of constant amount originating in the bone marrow stroma, coupled with an inhibitor of varying concentration derived from maturing hematopoietic bone marrow elements. The result of this interaction is bone marrow homeostasis.

MATERIALS AND METHODS

Mice. Female CD₂-F₁ (BALB/c × DBA/2) mice, aged 9 to 11 weeks and weighing ~20 g, were obtained from M. A. Bioproducts, Bethesda, MD and used within 6 weeks of arrival. They were housed ten to a cage and were given chlorinated tap water and Purina Laboratory Chow ad libitum. In three separate experiments, 30 mice per point received cyclophosphamide (CY), 300 mg/kg (LD₅) intraperitoneally (IP), or saline. The results of the three experiments were similar, and the data were combined for presentation (analysis of variance).

Rats. Female LBN-F₁ (Lew × BN) rats, aged 9 to 12 weeks, were obtained from Harlan Sprague Dawley and used within 6 weeks of arrival. They were kept four to a cage and were given Purina Rat Chow and water ad libitum.

In three separate experiments, nine rats per point received CY, 175 mg/kg (LD₅) IP. To determine the timing of the appearance and magnitude of HSA after cytosine arabinoside (ara-C), rats received a single course of drug at high dose to induce total bone

marrow aplasia, followed by recovery of formed elements over time. Triplicate animals were exsanguinated daily, and HSA, HIA, and WBC count were determined.

Humans. In initial trials, 12, and in subsequent trials, a further 20 adult patients who had progressive incurable neoplasms but no detectable physiological defects and whose hematopoietic function was adjudged normal by peripheral blood examination and bone marrow biopsy and aspirate were treated with CY, 120 mg/kg intravenously (IV). The effect on DNA synthesis of the pretreatment sera of each was within normal range. All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent.

Sera collection. Mouse blood was obtained by exsanguination from replicate animals daily until WBC recovery after therapy. Rat blood was obtained in a similar fashion under metaphane anesthesia. Prior to therapy and three times a week until the peripheral WBC count stabilized, 10 mL of human whole blood were collected from each patient and allowed to clot. All sera were separated and stored immediately at -70 °C. At the end of the collection period, all sera were assayed simultaneously to determine the effect of the sequential sera on [³H]TdR incorporation by bone marrow cells in the assay system. Normal sera were obtained from 30 volunteers and pooled. When these 30 normal sera were assayed individually for their effects on ³H-thymidine [³H]TdR incorporation by bone marrow cells, each fell within the range of 1 SEM of the activity of the pool. The activity present in pretreatment and posttreatment sera of all species was compared with the activity of the normal control pool in each assay and reported as a percentage of normal.

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Scintillation assay. The effect of sera on [^3H]TdR incorporation by normal rodent bone marrow cells was measured by the scintillation counting technique. Control studies in the laboratory have demonstrated that the sum of activities of distinct stimulatory and inhibitory humoral factors that influence cell proliferation is measurable by this method (ref 6, unpublished control observations, July, 1972).

In these assays, 1.0 $\mu\text{Ci/mL}$ of culture of [^3H]TdR (specific activity, 1.9 Ci/mmol) was added to tubes in triplicate of RPMI 1640 medium and incubated with fresh bone marrow cells ($10^6/\text{mL}$) at 37°C in a 7% CO_2 -humidified atmosphere at a final volume of 2 mL. Sera to be evaluated were added to attain a concentration of 30%. After 18 hours of incubation, the cultures were terminated by immersion in ice, and the cellular contents of each tube were collected under vacuum on a glass-fiber filter. Each tube was washed with cold 0.9% NaCl solution, and the wash was added to the filter. Following three washings with 0.9% NaCl solution, the acid-insoluble materials on the filter were precipitated by three washings with cold 5% trichloroacetic acid and were then washed three times with absolute ethanol. The dry filter was transferred to a screw-top vial to which 6 mL of scintillation fluid was added, cooled, and counted in a liquid scintillation spectrometer. Results are expressed as actual counts per minute for each experiment.

Preparation of [^3H]TdR microautoradiographs. Aliquots of rodent femur and aspirated human bone marrow tumor cells were prepared and analyzed for labeling index (LI) by methods described previously.⁴

Aplasia model. In studies in the model of lethal bone marrow aplasia, groups of rats were given an ablative dose of BU, 35 mg/kg IP on day 0. If not rescued with syngeneic normal bone marrow, all animals died by day 13 with signs and symptoms of bone marrow failure.⁷ In syngeneic transplant experiments, normal femur bone marrow cells were incubated at 37°C in RPMI 1640 media with 15% autologous sera at a concentration of $5 \times 10^5/\text{mL}$ at a 6-mL vol (30×10^6) in each 25-cm² Falcon TC flask, with eight flasks per point. After incubation for 2 hours, the total supernate with nonadherent cells was aspirated, the cells washed in RPMI media, and the total cells were injected IV into a single animal, 8 per group, on day 1 after BU. Similarly, each animal from another group of eight received the total adherent cells contained in the flask after removal by gentle agitation and scraping with a rubber policeman. Similar numbers of control animals received RPMI-1640 cultured media. Following injection, each animal was followed with assay of WBC, HIA, and HSA. Animals that survived were observed for 100 days before being killed.

Agar assay for CFU-GM. Cell suspensions from normal rat femurs were cultured at a final concentration of 1×10^5 cells in quadruplicate 35 $\times$ 10-mm Falcon Petri TC dishes in 1 mL of 0.3% Difco-Bacto and Dulbecco's minimal essential medium (DMEM) supplemented with penicillin, streptomycin, glutamine, asparagine, and 15% normal rat serum, or replicate sera obtained before, during, and after treatment at daily intervals from groups of drug-treated rats. Agar cultures were maintained at 37°C in a humidified atmosphere containing 7% CO_2 and scored for number of colonies (>40 cells) on day 7. All colonies were removed from each agar plate by gentle capillary pipetting, transferred to glass slides, and stained with 0.5% orcein, buffered Wright's stain, and myeloperoxidase to evaluate the cell types present in individual colonies. Comparison of numbers of colonies in pretreatment sera and in sera throughout marrow recovery was made by Student's *t* test and the Wilcoxon rank sum test.

Long-term stroma cultures. Split femurs, obtained on day 8 after rats were treated with a lethal dose of BU, 35 mg/kg, were cultured in replicate in 25-cm² Falcon TC flasks with 15% normal rat sera and 85% DMEM in conditions similar to the agar assay,

with weekly partial decanting of supernate and refeeding. Supernates were sterilized through a 0.22- μm filter and frozen at -70°C until assayed simultaneously for DNA synthesis in rat bone marrow cells.

Short-term conditioned media. Replicate femurs harvested at 2-day intervals from rats treated with CY, 175 mg/kg, were split longitudinally with a scalpel and cultured in 5% normal rat sera and 85% DMEM in 25-cm² Falcon TC flasks at 37°C in 7% CO_2 at a volume of 10 mL. During 3 successive days, four cultures were terminated daily, the conditioned supernate (HSA-CM) was sterilized through a 0.22- μm filter, and the cultures were frozen for later simultaneous analysis for DNA stimulatory activity in the 18-hour scintillation and CFU-c assay.

Endotoxin screening. All sera and conditioned media used in these experiments were determined to be free of endotoxin by the limulus amoebocyte lysate assay.

Spleen colony assay. CFU-S were assayed by the method of Comas and Byrd,⁸ with the modification that assay rats received total body irradiation at a total of 900 rad split equally at hour 1 and hour 6 and delivered at a dose rate of 116 ± 3 rads/minute from a ^{136}Cs -Gammacell 40 radiation source.

Stem cell suicide assays. In growth stimulation assays and suicide experiments, replicate cultures were established in Dulbecco's minima essential medium (DMEM) at a concentration of marrow cells $5 \times 10^5/\text{mL}$ and incubated for 17 hours with either normal sera, HSA, or HIA, [^3H]TdR, (85 $\mu\text{Ci/mL}$; specific activity, 20 Ci/mmol) added for the last 1 hour of the 18-hour cultures. The cultures were then washed three times with cold thymidine solution (0.8 mmol/L), washed three times with media, and then each of eight replicate cultures was injected intravenously (IV) in animals on day 1 after irradiation. Cells cultured in DMEM with specific sera without suicide drugs and handled similarly served as controls in these studies.

Statistical analysis. Data were analyzed using Student's *t* test to compare control groups with suicide groups, analysis of variance to determine if groups could be combined, SE to evaluate percentage of kill by the method of Lord and colleagues⁹ for the in vitro and in vivo CFU-S assays, and the Wilcoxon rank sum test to compare DNA synthesis over time.

RESULTS

Correlation of bone marrow recovery and humoral factor kinetics after drug-induced aplasia. Bone marrow cells and sera were obtained sequentially through aplasia and recovery from 12 patients with normal hematopoiesis who received CY, 120 mg/kg over 48 hours (Fig 1). The LI of the granulocyte precursor compartment (myeloblasts, progranulocytes and myelocytes; proliferative pool, Pro) increased significantly in all, with the mean peak LI detected on day 9 following CY (200% of pretreatment values), then fell to less than pretreatment levels (75%) by day 25 at the time of bone marrow maturation [metamyelocytes, bands, polymorphonuclear lymphocytes (PMNs); nonproliferative, NonPro].

The growth activity of the Pro paralleled the presence of HSA, which achieved peak values of 200% of pretreatment sera on day 10, then declined to a nadir on day 20 (50%). This decay of HSA and increasing HIA was associated with a decrease in bone marrow proliferative activity occurring at the time of metamyelocyte differentiation, 5 days before granulocyte release and 12 days before recovery of normal levels of WBC.

Comparison of hematopoietic recovery and HSA induction after drug-induced bone marrow aplasia in three spe-

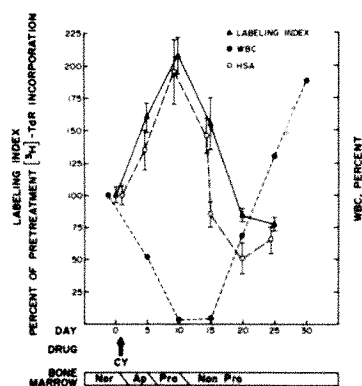


Fig 1. The mean WBC count (●), labeling index (▲), and effect of sequentially obtained sera on 18-hour normal bone marrow $[^3\text{H}]$ -thymidine ($[^3\text{H}]\text{TdR}$) incorporation (○) from 12 patients treated with cyclophosphamide (CY), 120 mg/kg. Vertical bars represent ± 1 SEM.

cies. Absolute bone marrow aplasia was induced in three species (mouse, rat, human) by a single comparable dose of CY, and the temporal relationship of hematopoietic recovery and HSA induction was monitored in each and compared (Fig 2). Sequential sera were collected daily from $\text{CD}_2\text{-F}_1$ mice (30/point) that had received CY 300 mg/kg IP (LD_{50}), from LBN rats (9/point) that had been given CY 175 mg/kg IP (LD_{50}), and sequentially from 20 patients with malignancy but with normal bone marrows who had received CY 120 mg/kg IV on day 0 of therapy. The effect of these serial sera on $[^3\text{H}]\text{TdR}$ incorporation into the DNA of autologous bone marrow cells was measured in vitro in simultaneous replicate cultures, and the results were expressed as the mean percentage of their respective day 0 control value ± 1 SEM (Fig 2).

These data demonstrated the temporal relationship of bone marrow regeneration and associated peak detection of

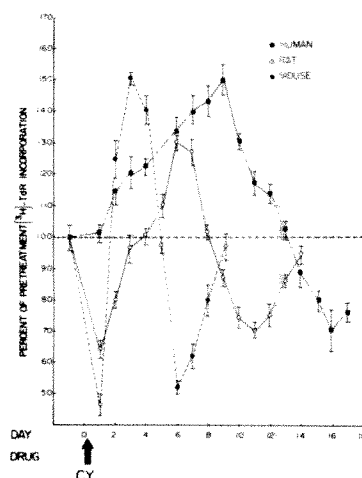


Fig 2. Correlation of humoral stimulating activity (HSA) and hematopoietic recovery time after cyclophosphamide (CY)-induced aplasia in mouse, rat, and human. All animals received comparable doses of CY. After an initial fall in activity, HSA reached maximum levels on day 3 in mouse (●), day 6 in rat (○), and day 9 in human (▲), then fell to levels below pretreatment. These oscillations were the reciprocal of the WBC count.

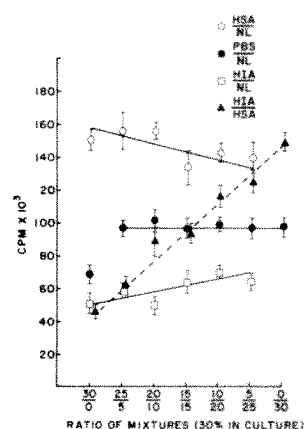
HSA and HIA induction following bone marrow aplasia induced by a single comparable dose of drug in the three species. The ratio was 1:2:3 in mouse, rat, and human, respectively.

Serum mixing experiments. To determine the relative growth promoting activity present in the sera, pools of HIA, HSA, and normal sera were mixed at various concentrations and assayed using normal marrow cells (Fig 3). Normal sera at 30% supported growth well, whereas concentrations as low as 5% HIA or HSA mixed with normal sera produced a significant effect on DNA synthesis. When HIA and HSA were mixed in equal proportions, the activity produced equaled that of normal sera. The lines were constructed by best-fit analysis.

Correlation of HSA and degree of aplasia. Rats were given incremental amounts of drugs to maximally tolerated doses: CY, 25 to 200 mg/kg IP; ara-C, 50 to 400 mg/kg every 8 hours $\times 6$ subcutaneously; and BU, 5 to 30 mg/kg IP (Fig 4). There was a linear relationship of drug dose and the effect of sera obtained on day 6 after each drug on DNA synthesis of cultured bone marrow cells. The peak quantity of HSA induced related directly to the dose of drug and resultant amount of bone marrow depression.

Source of humoral factors: media conditioned by bones. Aliquots of the cell-free conditioned media (CM) taken daily for 3 days from the 24-, 48-, and 72-hour cultures of split femurs from replicate rats killed at 2-day intervals after treatment with CY, 175 mg/kg, demonstrated an effect in the DNA scintillation assay that paralleled the measured HSA in sera. Day 4 femurs containing early recovery proliferative phase bone marrow elements, as measured by a rising LI and progranulocytic morphology, elaborated factors over 3 days which promoted DNA synthesis that was 155% greater than that of normal bones (Fig 5). Bones harvested on day 6 of CY and established in culture produced increasing amounts of HIA, with subnormal DNA synthesis induced by the 72-hour CM. In the experiment carried through the period of bone marrow recovery and "hypermaturation," the media from bones cultured on day 10 at the time of WBC recovery and granulocytosis markedly depressed DNA synthesis, reflecting the presence of HIA. At the time of reestablished bone marrow homeostasis, media conditioned by femurs from treated animals, which then contained

Fig 3. Effect on DNA synthesis in normal bone marrow cells of mixing normal sera with phosphate-buffered saline (PBS) (●), humoral stimulating activity (HSA) (○), humoral inhibitory activity (HIA) (□), and HSA with HIA (▲), in various proportions. The final concentration of serum is 30%. Vertical lines are ± 1 SEM.



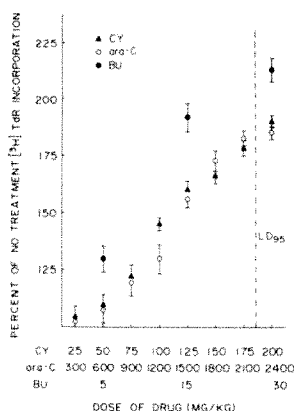


Fig 4. The dose-response of humoral stimulating activity (HSA). The quantity of HSA induced by incremental doses of the bone marrow suppressing drugs cyclophosphamide (CY) (Δ), cytosine arabinoside (ara-C) ($\square$), and busulfan (BU) ($\bullet$) is linear, as measured by the effect of sera on DNA synthesis in normal bone marrow.

normal marrow elements and cellularity, influenced DNA synthesis in a manner similar to that of normal controls. The curves of DNA synthesis generated by these CM correlated directly with the DNA synthesis induced by the serum obtained on day 1 of each culture set.

These results demonstrate that femurs obtained during aplasia and subsequent recovery elaborate soluble substance(s) during 72-hour culture that stimulate and inhibit DNA synthesis. Concomitantly, similar humoral activities are detected in autologous sera, suggesting that the source of these serum factors is likely bone and/or bone marrow cells.

Stimulatory media conditioned by long-term stromal cultures. Stromal cells from marrows of rats lethally treated with BU were established in culture (Fig 6). Adherent cells grew to a confluent monolayer in 2 to 3 weeks and supported the growth of increasing numbers of sessile, round cell colonies on stalks containing macrophages and monocytes. CM from replicate flasks cultured with 15% normal sera in two experiments stimulated the growth of hematopoietic cells to 120%, 163%, and 147% above normal serum on days 7, 14, and 21, respectively. Replicate cultures maintained through week 9 continued to condition media to stimulatory levels >130% of control media and sera. Cultures of normal bone marrow adherent cells, with nonadherent cells removed, stimulated growth of hematopoietic cells to 130% of normal by day 14 of culture (Fig 6).

These data demonstrate the viability of stromal cells after in vivo lethal doses of BU, the production of HSA by such

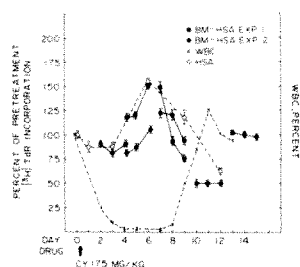


Fig 5. The conditioning of media by femurs of cyclophosphamide (CY)-treated rats. Femurs harvested from animals before treatment and when recovering from drug-induced aplasia, and cultured at 2-day intervals produced conditioned media ($\bullet$, $\blacksquare$) both stimulated and inhibited DNA synthesis in a similar fashion to sera ($\circ$) obtained sequentially from the same rats. Vertical bars are ± 1 SEM.

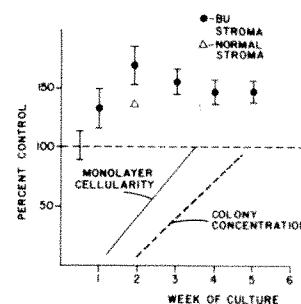


Fig 6. Stimulation of hematopoietic cell proliferation by media conditioned by stromal cells from busulfan (BU)-treated rats. Supernates from stromal cultures were decanted weekly and assayed for DNA synthesis stimulation in marrow hematopoietic cells ($\bullet$) and compared with those in simultaneous cultures without cells (-----). Bars ($\pm$) are the SEM of two experiments; Δ indicates the stimulation induced by conditioned media from normal bone marrow adherent cells depleted of hematopoietic cells, assayed at 2 weeks. Solid line, degree of confluency of the growing monolayer of cells; dotted line, cellularity of the epithelial, mononuclear, and adipose cell colonies growing as sessile pedunculated pods on the monolayer.

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BU model of aplasia. The lethal bone marrow ablation induced by >24 mg/kg of BU in the rat and the amelioration of that aplasia with infused syngeneic bone marrow cells⁷ provides a useful model to study mechanisms of hematopoietic reconstitution (Fig 7). A series of experiments involving bone marrow rescue to relate dose and source of cells with restoration of bone marrow function and production of HSA and HIA was conducted. In this model of aplastic anemia, HSA increases linearly to maximal levels at the time of most profound aplasia prior to the death of the animal from the sequelae of bone marrow failure. In the studies reported here, 250 rats were given BU, 35 mg/kg on day 0. Twenty-four hours after BU, each of one-third of the animals received IV

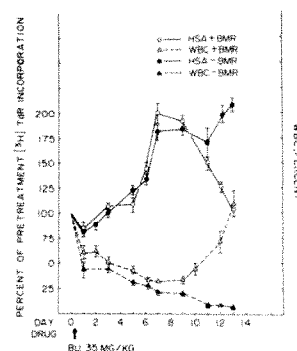


Fig 7. Busulfan (BU) model of bone marrow aplasia and rescue. Groups of eight animals received BU 35 mg/kg intraperitoneally on day 0. Four animals in each group were given 30×10^6 autologous nonadherent bone marrow cells intravenously on day 1 (BMR+); the remainder received media alone (BMR-). WBCs (Δ) and humoral stimulating activity (HSA) ($\circ$) of the BMR+ were contrasted with the WBCs ($\blacktriangle$) and HSA ($\bullet$) of the BMR- animals. All BMR- animals died by day 13, whereas those rescued survived and were killed at 100 days. Engraftment of donated autologous nonadherent bone marrow cells was associated with a decline in HSA as the bone marrow matured.

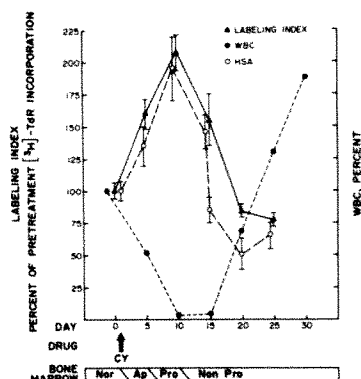


Fig 1. The mean WBC count (●), LI (▲), and effect of sequentially obtained sera on 18-hour normal bone marrow [^3H]-thymidine [^3H]TdR incorporation (○) from 12 patients treated with cyclophosphamide (CY), 120 mg/kg. Vertical bars represent ± 1 SEM.

cies. Absolute bone marrow aplasia was induced in three species (mouse, rat, human) by a single comparable dose of CY, and the temporal relationship of hematopoietic recovery and HSA induction was monitored in each and compared (Fig 2). Sequential sera were collected daily from $\text{CD}_2\text{-F}_1$ mice (30/point) that had received CY 300 mg/kg IP (LD_{50}), from LBN rats (9/point) that had been given CY 175 mg/kg IP (LD_{50}), and sequentially from 20 patients with malignancy but with normal bone marrows who had received CY 120 mg/kg IV on day 0 of therapy. The effect of these serial sera on [^3H]TdR incorporation into the DNA of autologous bone marrow cells was measured in vitro in simultaneous replicate cultures, and the results were expressed as the mean percentage of their respective day 0 control value ± 1 SEM (Fig 2).

These data demonstrated the temporal relationship of bone marrow regeneration and associated peak detection of

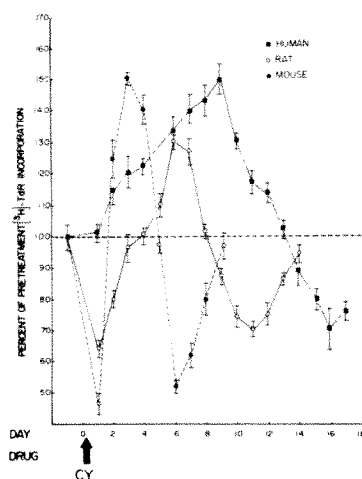


Fig 2. Correlation of humoral stimulating activity (HSA) and hematopoietic recovery time after cyclophosphamide (CY)-induced aplasia in mouse, rat, and human. All animals received comparable doses of CY. After an initial fall in activity, HSA reached maximum levels on day 3 in mouse (●), day 6 in rat (○), and day 9 in human (■), then fell to levels below pretreatment. These oscillations were the reciprocal of the WBC count.

HSA and HIA induction following bone marrow aplasia induced by a single comparable dose of drug in the three species. The ratio was 1:2:3 in mouse, rat, and human, respectively.

Serum mixing experiments. To determine the relative growth promoting activity present in the sera, pools of HIA, HSA, and normal sera were mixed at various concentrations and assayed using normal marrow cells (Fig 3). Normal sera at 30% supported growth well, whereas concentrations as low as 5% HIA or HSA mixed with normal sera produced a significant effect on DNA synthesis. When HIA and HSA were mixed in equal proportions, the activity produced equaled that of normal sera. The lines were constructed by best-fit analysis.

Correlation of HSA and degree of aplasia. Rats were given incremental amounts of drugs to maximally tolerated doses: CY, 25 to 200 mg/kg IP; ara-C, 50 to 400 mg/kg every 8 hours $\times 6$ subcutaneously; and BU, 5 to 30 mg/kg IP (Fig 4). There was a linear relationship of drug dose and the effect of sera obtained on day 6 after each drug on DNA synthesis of cultured bone marrow cells. The peak quantity of HSA induced related directly to the dose of drug and resultant amount of bone marrow depression.

Source of humoral factors: media conditioned by bones. Aliquots of the cell-free conditioned media (CM) taken daily for 3 days from the 24-, 48-, and 72-hour cultures of split femurs from replicate rats killed at 2-day intervals after treatment with CY, 175 mg/kg, demonstrated an effect in the DNA scintillation assay that paralleled the measured HSA in sera. Day 4 femurs containing early recovery proliferative phase bone marrow elements, as measured by a rising LI and progranulocytic morphology, elaborated factors over 3 days which promoted DNA synthesis that was 155% greater than that of normal bones (Fig 5). Bones harvested on day 6 of CY and established in culture produced increasing amounts of HIA, with subnormal DNA synthesis induced by the 72-hour CM. In the experiment carried through the period of bone marrow recovery and "hypermaturation," the media from bones cultured on day 10 at the time of WBC recovery and granulocytosis markedly depressed DNA synthesis, reflecting the presence of HIA. At the time of reestablished bone marrow homeostasis, media conditioned by femurs from treated animals, which then contained

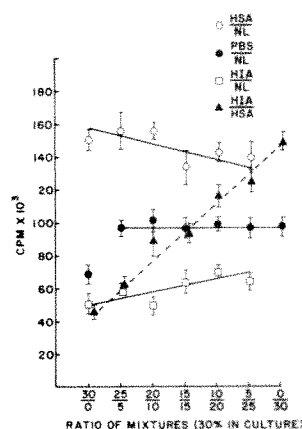


Fig 3. Effect on DNA synthesis in normal bone marrow cells of mixing normal sera with phosphate-buffered saline (PBS) (●), humoral stimulating activity (HSA) (○), humoral inhibitory activity (HIA) (□), and HSA with HIA (▲), in various proportions. The final concentration of serum is 30%. Vertical lines are ± 1 SEM.

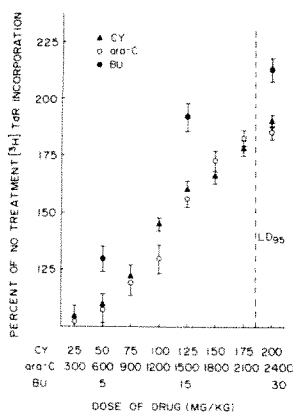


Fig 4. The dose-response of humoral stimulating activity (HSA). The quantity of HSA induced by incremental doses of the bone marrow suppressing drugs cyclophosphamide (CY) (▲), cytosine arabinoside (ara-C) (○), and busulfan (BU) (●) is linear, as measured by the effect of sera on DNA synthesis in normal bone marrow.

normal marrow elements and cellularity, influenced DNA synthesis in a manner similar to that of normal controls. The curves of DNA synthesis generated by these CM correlated directly with the DNA synthesis induced by the serum obtained on day 1 of each culture set.

These results demonstrate that femurs obtained during aplasia and subsequent recovery elaborate soluble substance(s) during 72-hour culture that stimulate and inhibit DNA synthesis. Concomitantly, similar humoral activities are detected in autologous sera, suggesting that the source of these serum factors is likely bone and/or bone marrow cells.

Stimulatory media conditioned by long-term stromal cultures. Stromal cells from marrows of rats lethally treated with BU were established in culture (Fig 6). Adherent cells grew to a confluent monolayer in 2 to 3 weeks and supported the growth of increasing numbers of sessile, round cell colonies on stalks containing macrophages and monocytes. CM from replicate flasks cultured with 15% normal sera in two experiments stimulated the growth of hematopoietic cells to 120%, 163%, and 147% above normal serum on days 7, 14, and 21, respectively. Replicate cultures maintained through week 9 continued to condition media to stimulatory levels >130% of control media and sera. Cultures of normal bone marrow adherent cells, with nonadherent cells removed, stimulated growth of hematopoietic cells to 130% of normal by day 14 of culture (Fig 6).

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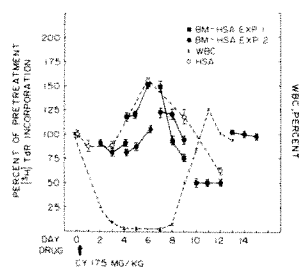


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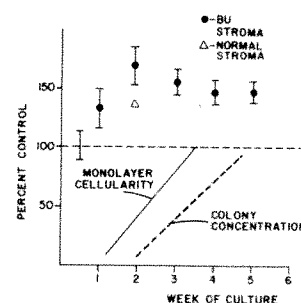


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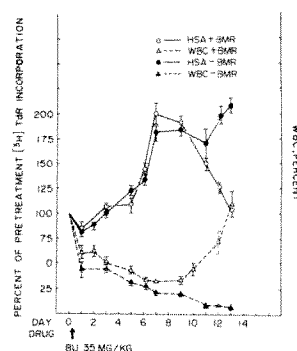


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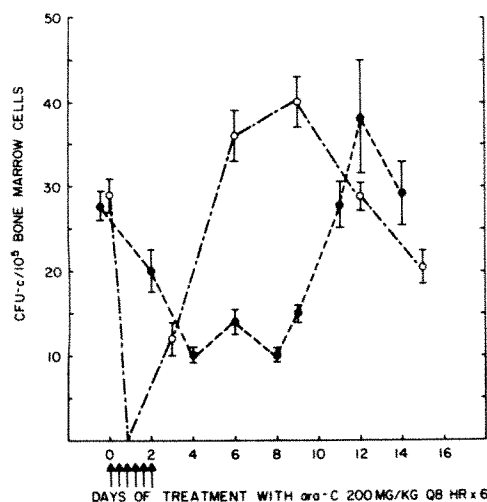


Fig 8. Effect of sequential sera obtained before and after cytosine arabinoside (ara-C), 200 mg/kg every 8 hours $\times$ 6 on bone marrow CFU-GM (O—O). The growth is the reciprocal of the WBC count (●—●). Vertical lines are $\pm$ 1 SEM.

the total nonadherent autologous nucleated bone marrow cells from a single flask of replicate cultures initiated at 30×10^6 cells (bone marrow rescue, BMR+). Each animal in a second group received the total population of adherent cells from a single flask after depopulation of the nonadherent cells (BMR-). Each animal in a third group was given culture media. All were monitored for fluctuations of WBCs, and HSA and HIA were gathered every other day from four rats of each group. Granulocyte counts in all animals dropped rapidly to 0 by day 7, whereas the number of absolute lymphocytes fell to 25% of initial values.

HSA levels rose gradually in both BMR+ and BMR- groups to ranges between 175% and 200% of pretreatment values by day 7. The ultimate value prior to death in the BMR- group reached 210% on day 13. Net detectable HSA in BMR+ animals declined by day 9 as the bone marrow cellularity increased with all proliferative hematopoietic elements present and returned to pretreatment levels at the time of WBC recovery.

Bone marrow aspirates of the animals not given bone marrow support (BMR-) demonstrated marked bone marrow hypoplasia by day 7, and all animals were dead with hemorrhage and/or infection by day 13. In contrast, the WBCs in the BMR+ group began to recover by day 10 and

reached pretreatment counts by day 12 through 14. The cellular differential, primarily lymphocytes in the unperturbed state, now revealed granulocytes (75%) by day 13 following autologous rescue. These animals rescued from bone marrow aplasia survived until killed on day 100.

Effect of HSA and HIA on CFU-GM. Replicate normal LBN-F₁ rats given ara-C, 200 mg/kg every 8 hours $\times$ 6, were exsanguinated at intervals; their WBCs were counted, and the sera were stored. After WBC recovery from aplasia, the collected sera were plated at 15% in agar in replicate cultures containing 1×10^5 bone marrow cells, with cell concentrations and media similar to those routinely used in the 18-hour [³H]TdR assay of DNA synthesis. Colonies of >40 cells visible in four replicate dishes counted on day 7 of agar culture were identified as CFU-GM by peroxidase staining and morphology with Wright's stain. Pretreatment sera supported the growth of 28 ± 2 colonies, whereas no colonies were present in sera containing ara-C; 36 ± 3 colonies grew in sera obtained on day 6, and 41 ± 3 grew in sera of day 9 during the time of bone marrow aplasia and early recovery. Only 21 ± 2 colonies grew in sera collected at the time of WBC recovery and a mature bone marrow (Fig 8). These counts were within the range of linearity of this assay in our laboratory and differed significantly from each other ($P < .05$). Similar results were obtained in two subsequent experiments.

Effect of HSA and HIA on CFU-S. The results of 6 experiments, 3 with and 3 without [³H]TdR suicide, each experiment having 8 recipient rats for each sera in replicate cultures, were combined (analysis of variance) (Table 1). Normal bone marrow cells were cultured with normal sera, HSA, and HIA for 18 hours and were then injected into irradiated recipient rats, with quantitation of spleen colonies on day 8. In the six studies without a suicide component, HSA increased the number of colonies by 50% (Table 1, $P < .001$). Most unexpected was the stimulation of spleen colony formation by HIA, a reagent previously determined to be inhibitory to growth of hematopoietic cells in both the DNA scintillation and the CFU-GM assay. This paradoxical stimulatory action of HIA was confirmed in the [³H]TdR suicide experiments. Although the number of CFU-S was not significantly reduced by [³H]TdR in either normal or HSA cultures (10%), culture with HIA produced a significant reduction in total colonies formed (33%, $P < .005$), demonstrating that there had been movement of CFU-S cells into cycle in the presence of HIA.

Table 1. Effect of Humoral Regulators on Growth of Stem Cells

Sera	CFU-S	Normal (%)	[³ H]TdR Suicide			
			CFU-S Control	Normal (%)	[³ H]TdR	CFU-S in Cycle (%)
NL	$18.0 \pm 2.5^*$	100†	19.0 ± 1.9	100	17.0 ± 2.1	$10.5 \pm 13.9‡$
HSA	26.4 ± 1.9	146§	28.5 ± 1.6	150§	25.8 ± 2.3	9.6 ± 9.5
HIA	32.1 ± 2.9	178§	29.4 ± 3.1	149§	19.8 ± 3.1	$32.7 \pm 14.1§$

[³H]TdR, ³H-thymidine; NL, normal sera; HSA, humoral stimulatory activity; HIA, humoral inhibitory activity.

*SEM, the mean $\pm$ SE of 24 assays.⁶¹

†Normal (%) represents the mean percentage of normal sera compared using Student's two-tailed *t* test.⁶¹

‡SEM calculated by the method of Lord and colleagues.⁹

§ $P < .001$.

DISCUSSION

Periodicity of hematopoiesis. After drug ablation of cells in the proliferative phase of the bone marrow, the eventual depletion of the residual mature cells that prevent major fluctuation in circulating blood elements results in marrow aplasia and pancytopenia. With dissipation of these buffers, the oscillating forces that drive marrow reconstitution are readily measurable in the blood (Figs 1 and 2).

HSA, directly related to the rate of marrow regrowth, reaches maximal values at the time of full proliferation of regenerating cells. Subsequently, with the enlarging nonproliferative phase of marrow recovery, HIA becomes the dominant humoral regulator. Whether HSA and HIA act independently, are a summation of activities both local and distant from the bone marrow, are a spillover of excess amounts of locally acting forces, or are unrelated to hematopoiesis has been scrutinized.

These studies using sera as reagents are imprecise at best, since cytotoxic agents that induce marrow aplasia in the intact animal also damage other growing tissues. During the period of cell recovery, it is reasonable to assume that the blood contains myriad pleiotrophic and tissue-specific growth promoters mixed with synergistic proteins, likely multiple, that are responsible for the restoration of the cellular elements and microenvironment of the bone marrow. We lump these combined activities as HSA and HIA, likely pluripoiectins from multiple sources that reflect the global cytotoxic effects of drug and the summation of all regenerative forces active in the intact animal. The amplitude of HSA and HIA relates directly to the dose of drug and the resultant degree of aplasia induced, although the regeneration time of bone marrow recovery is unchanged and is species-specific.

Humoral regulators. Lord and co-workers and Wright and colleagues identified distinct local activities in the adherent layer of cultures of regenerating bone marrow that independently stimulate or inhibit hematopoiesis with a balanced oscillation,¹⁰⁻¹² whereas Frindel and colleagues defined the role of diffusible serum factors that regulate CFU-S growth and proliferation at a distance from their source of production.¹³⁻¹⁵ Complicating the interpretation of humoral data is the variable presence in serum of factors such as endotoxin^{16,17} that rapidly stimulate increased synthesis and release of colony-stimulating activity (CSA) by mononuclear cells.¹⁸

Stroma cultures derived from animals with hematopoiesis ablated by BU have morphology consistent with that described by Dexter and co-workers and Bentley and Foidart^{19,20} but do not produce CFU-C or CFU-S.^{7,21} This transplanted stroma produces HSA without inhibition (Fig 6), mimicking HSA kinetics as measured in the BU-treated rat unrescued by donated bone marrow (Fig 7). Verification that the stromal cell is the source of HSA comes from other laboratories as well.²²⁻²⁵ Cross-reactivity between HSA and mouse L cell colony-stimulating factor (CSF) has been demonstrated,²⁶ and it is likely that HSA is a composite of a number of CSFs and other cell- and tissue-specific substances. The relative decrease with marrow recovery may reflect down-regulation of active sites for CSA and not relate to an actual reduction of available stimulating factor.²⁷

During recovery from induced aplasia, HSA is balanced by HIA, the two producing a curve of equal duration and opposite direction and the reciprocal of the WBC. Bone marrow extracts of a short-acting inhibitory activity that block the entry of CFU-S into cycle^{28,29} may be produced by bone marrow macrophages.^{30,31} Candidates for this function are the glycoproteins lactoferrin (LF), transferrin (TF), acidic isoferitin (AIF),^{32,33} and type E prostaglandins (PGE II).³⁴⁻⁴⁰

Aplasia model. The BU model of lethal bone marrow aplasia serves as a bioassay for the effect of restoration of function on net humoral activities by selected infused cell populations separated from whole bone marrows (Fig 7). Sera obtained sequentially during the period of aplasia have increasing amounts of HSA, with peak activity at the time of profound aplasia prior to death. Such maximal levels of HSA have also been detected in patients with idiopathic aplastic anemia.⁴¹

Detectability of HSA in the BU model is countered by rescue of lethally treated rats with nonadherent cells obtained from short-term bone marrow cultures. These hematopoietic elements produce increasing HIA over time following injection until balanced bone marrow reconstitution occurs. In contrast, the infusion of adherent "stromal" cells alters neither the maximal level of stimulator nor the fatal outcome. HIA is derived from the nonadherent hematopoietic cells, occurs with engraftment and maturation of all formed elements of the rescue marrow, and is apparent before release of cells into the peripheral blood.

Effect on stem cells. The effects of stimulators and inhibitors from varied sources on bone marrow precursor cell growth and differentiation have been measured. The increased proliferation of CFU-S following treatment of mice with phenylhydrazine is accompanied by an increase in stimulator and a simultaneous decrease in inhibitor production. The two distinct factors¹⁰⁻¹² alternately block each other's synthesis in response to a signal for more or less production from the progenitor cell compartment⁴²—data supporting the theory that activities of short-range substances contained in the marrow locally regulate stem cell cycle.⁴³⁻⁴⁵ In contrast, investigating the cause of movement away from self-renewal to differentiation to committed stem cells, Guignon and colleagues and Sainteny and co-workers found that inhibitor production after treatment with ara-C channels stem cells into an altered erythroid-granulocytic balance^{46,47} by factors acting at a distance.⁴⁷⁻⁴⁹

In our progenitor cell cultures, HSA produced increased growth of stem cells. In addition, there was unexpected stimulation of growth of CFU-S by HIA, a reagent determined to be inhibitory to growth of hematopoietic cells in both the DNA scintillation and the CFU-GM assay. This paradoxical stimulatory action was confirmed in the [³H]TdR suicide experiments, with movement of CFU-S cells into cycle. These findings suggest that HIA is short-lived and that the HIA-containing sera obtained during bone marrow maturation contained maximal HSA as well. HSA is effectively blocked for the brief time of the other assay systems by the short-acting inhibitory substance in high concentration. Constantly available only in vivo, in 18-hour cultures un replenished HIA apparently disappears and HSA

acts unopposed. This result supports the concept of a long-lived stimulator whose constant presence is buffered and neutralized, as in the mixing experiments, by a short-acting local but powerful inhibitory factor that increases with maturation of hematopoietic elements in the bone marrow.

The growth effect of stimulators on CFU-S but their inconsistent movement into cell cycle^{50,51} suggests the presence of a pool of progenitor cells with a heterogeneous response to growth promotion v differentiation or with a random availability for growth promotion toward pluripotent cells. The [³H]TdR suicide experiments and our clinical experience with sequential therapy timed at maximal bone marrow regrowth⁵²⁻⁵⁵ offer little evidence for significant killing of CFU-S by cytotoxic cell cycle-dependent drugs in vitro. Either such drugs have little direct effect on stimulated stem cells²⁴ or the site of action of these poietins is a differentiated or committed and expendable subset of the pluripotent cell population.⁵⁶

Hematopoiesis model. The demonstration of a sine wave during marrow ablation and recovery is consistent with the concept of two long-range regulators of hematopoiesis^{51,57,59} and with the theory that postulates two distinct local and counter-balancing activities within the bone marrow (Fig 9).^{10,42} HIA fits the category of short-term and short-range inhibition, whereas HSA has long-term activity. Both are released into the peripheral blood. Although the data of Lord and colleagues^{43,44} support an ebb and flow of both HSA and HIA, with reciprocal activity, our results in the BU model with rescue suggest instead the presence of a constant stimulator of stromal source countered periodically by a short-lived locally acting inhibitor, elaborated by maturing hematopoietic elements. That HSA directly stimulates the stem cell pool as a long-range regulator is supported by our stem cell assays and by evidence from other laboratories as well that aplastic animals elaborate long-range humoral regulators of stem cell growth.⁶⁰

This model of perturbed hematopoiesis (Fig 9) is most consistent with data derived from our sequential studies. Administration of drug X effectively eliminates all cells in the bone marrow that are in cell cycle. As the residual mature bone marrow cells decrease, HSA, comprised of a number of glycoproteins with CSA capability, increases. Emanating from the stroma, unopposed HSA acts on quiescent stem cells which are capable of entering at random either a self-renewal mode or differentiation to committed progenitors. At maximum levels of HSA on day 8 through 10

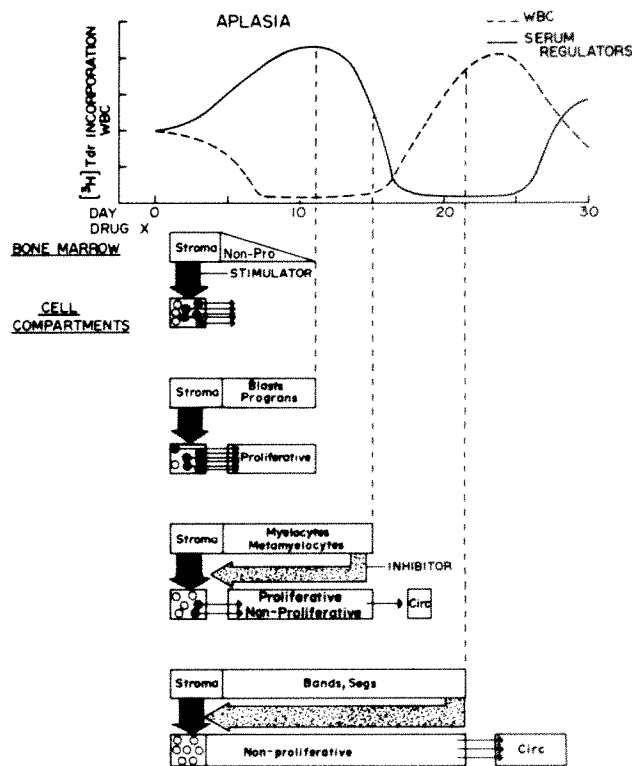


Fig 9. Model of hematopoiesis.

after cytotoxic drug, the bone marrow is totally proliferative. HSA continues to be produced at a constant amount, but its net measurable amplitude decays as HIA, a short-acting but powerful inhibitor derived from bone marrow cells in the maturing hematopoietic compartment, interacts directly with HSA to counter its activity. As the maturation compartment enlarges and net inhibition ensues, mature elements are released into the peripheral blood while access from the stem cell pool to the proliferative compartment is quenched. Ultimately, after this overshoot, the oscillation is dampened by loss of mature cells, and homeostasis is restored.

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Proplatelets and Stress Platelets

By M. Tong, P. Seth, and D.G. Fenington

The process of platelet formation by the fragmentation of megakaryocyte pseudopodia, termed proplatelets, demonstrable in the marrow sinusoids is poorly understood. "Stress" platelets produced under conditions of stimulated platelet production differ from normal circulating platelets with respect to volume and a number of functional characteristics. To clarify the relationship of stress platelets to proplatelets, rats were injected with heterologous platelet antiserum. Nondiscoid platelet forms, some characteristically beaded in appearance, strongly resembling bone

marrow proplatelets, can be recovered in the circulation of normal rats. During the early period of recovery from acute thrombocytopenia, there was a substantial increase in the proportion of these elongated platelets in the citrated platelet rich plasma. Exposure to EDTA rendered them spherical. Circulating proplatelets may contribute significantly to the prompt increase in platelet volume during recovery from acute thrombocytopenia at a time prior to significant increase in megakaryocyte size and ploidy.

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PLATELETS PRODUCED during recovery from acute thrombocytopenia show a striking increase in mean volume^{1,2} which under steady state conditions of increased production does not depart from a log normal size distribution.³ Odell and colleagues⁴ showed the appearance of large or "giant" platelets as early as 12 hours after the induction of thrombocytopenia well before any increase in the rate of platelet production could be expected. Although they are undoubtedly young platelets freshly produced by megakaryocytes in response to the acute stress of thrombocytopenia, caution must be exercised in equating them to normal young platelets. Hence, we adopted the term "stress platelets" to describe those platelets circulating during this period of accelerated platelet production.

A characteristic sequence of events in the bone marrow megakaryocytes following the administration of anti-platelet serum (APS) has been documented by a number of investigators. The first recognizable change within 2 to 6 hours in rodent bone marrow is the increase in the number of small acetylcholinesterase-positive diploid cells.^{5,6} This is followed in 18 hours of thrombocytopenia by an increase in the endomitotic index of the megakaryoblasts and subsequently an increase in the total number of immature megakaryocytes, most prominent at 40 hours.⁴ By 48 hours, there is a striking increase in the size of megakaryocytes in the marrow smear⁷; this is associated with an increase in the modal ploidy of the megakaryocytes from 16n to 32n that reaches a peak at 48 hours but returns to normal by 4 to 5 days.⁸ This increase in modal ploidy was also demonstrated by Levin and colleagues,⁹ who studied the effect of thrombocytopenia on ploidy distribution in megakaryocyte colonies obtained from soft agar culture. A significant number of 64n and even 128n cells is also present at 48 hours, whereas such cells are not readily detectable in the normal megakaryocyte colonies. Some investigators have suggested that this change in ploidy may determine platelet numbers¹⁰ and volume.¹¹⁻¹³ Although

this could be true in the "steady state," however, it cannot account for the early macrothrombocytosis observed by Odell and co-workers⁴ before any increase in endomitosis or in ploidy.

The presence of large intrasinusoidal cytoplasmic processes of megakaryocytes with relatively organelle-free constricted segments was demonstrated by Becker and de Bruyn¹⁴ and Radley and Scurfield¹⁵ using scanning electron microscopy, and these were termed proplatelets by Becker and de Bruyn. This led us to speculate that large circulating megakaryocyte fragments or proplatelets may contribute to the macrothrombocytosis that occurs in situations of altered platelet production; in many situations, their presence may have been obscured by the use of EDTA as an anticoagulant leading to the sphering of such proplatelet forms. This hypothesis has been tested in the rat.

MATERIALS AND METHODS

General. Adult Sprague-Dawley rats (250 to 400 g body weight) were used. Adult rabbits were immunized with a purified whole platelet preparation from rats by subcutaneous injections according to the schedule described by Vaitukaitis and co-workers¹⁶ to produce APS. The dosage (150–200 μ L/100 g per rat) of the APS (RBC- and WBC-absorbed and heat-inactivated) had been titrated in each batch to produce ~90% reduction of pretreatment platelet count at 24 hours after intravenous (IV) injection. Ether-anesthetized rats were injected with the appropriate single dose of APS through the jugular vein. Whole blood platelet counts were performed before and after APS treatment on EDTA-anticoagulated blood samples taken from the jugular vein. With 1% ammonium oxalate as diluent, a 1:20 dilution was made from the blood sample and platelets were enumerated under phase-contrast microscopy in a counting chamber. The anesthetized animals were killed by exsanguination at various intervals during a 168-hour period after APS treatment. Blood samples were harvested by inferior vena cava (IVC) or aortic puncture and were collected into either one of the following anticoagulants: ACDE₁—100 mL of acid-citrated dextrose containing 20 μ g/mL of prostaglandin E₁ (PGE₁), pH 6.7, 1 mL of freshly thawed ACDE₁ to 5 mL of blood; or EDTA dipotassium salt, 1.5 mg/1 mL of blood. Platelet-rich plasma (PRP) was prepared by centrifugation, 130 g for 15 minutes at 22°C in a swing-out rotor. At this speed, the average platelet recovery in the PRP was always >65%, with total RBC and WBC contamination <0.1%.

Platelet volume. Platelet counting and sizing were performed on PRP using a Coulter counter model ZF particle counter with a 50 $\times$ 60- μ mol/L aperture, coupled to a 2,000 channel multichannel analyzer. Latex spheres of 1.15- and 2.03- μ m diameter were used for calibration. No correction was introduced for the departure of

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platelet shape from that of the reference sphere. Either ACDE₁ or EDTA was used as anticoagulant in the PRP. Final platelet concentration was adjusted to between 100 and $180 \times 10^9/L$ to minimize error due to simultaneous passage of multiple platelets through the aperture. Mean platelet volume (MPV) was calculated from the formula:

$$MPV = \sqrt{(\text{median}^3/\text{mode})},$$

according to Martin and colleagues.¹⁷ Volume measurement was routinely carried out 1 hour after blood collection—the time taken to prepare PRP plus 20 minutes standing at 22 °C, allowing platelets to recover in PRP from the trauma of collection.

Platelet morphology. The morphology of platelets was observed by phase-contrast microscopy. Differential counts on platelet morphology in PRP were performed at $\times 250$ magnification with the platelets drifting slowly between the coverslip and ordinary glass slide. The number of platelets counted in each sample ranged from 1,700 to 2,400 in normal rats to 200 to 900 in APS-treated rats.

Platelets were classified into the following forms: (a) discoid; (b) elongated—length greater than three times the width; (c) beaded forms—showing two or three well-formed cytoplasmic masses separated by constriction bands or slender cytoplasmic threads; and (d) strings—very long platelet processes, showing more than three beads and frequently folding upon themselves due to their excessive length.

For electron microscopy, PGE₁ at a dose of $0.5 \mu\text{g}$ was injected into the jugular vein of ether-anesthetized rats prior to blood harvesting. PRP was prepared from vena cava blood collected into syringes loaded with ACDE₁ and glutaraldehyde (final 0.1%) for prefixation. Platelet buttons were further fixed in 3% glutaraldehyde in 0.1 mol/L of sodium cacodylate buffer and postfixed in 1% osmium tetroxide, dehydrated in graded acetone, and then embedded in Spurr's resin. Sections were stained with uranyl acetate and lead citrate.

For scanning electron microscopy, nucleopore membrane filters (pore size $0.6\text{-}\mu\text{m}$ diameter) were used to filter the prefixed platelets from the PRP. The platelet-containing filter was then fixed in osmium and dehydrated in graded ethanol in the usual manner. It was then subjected to critical point drying by CO₂ and mounted on to stubs for gold coating. Statistical analysis was carried out by Student's *t* test unless otherwise specified.

RESULTS

Platelet counts. Figure 1 shows that at 24 hours after APS injection, the whole blood platelet count had dropped to $12.6\% \pm 5.5\%$ of the pretreatment value. Thereafter, the platelet count began to rise, being significantly above normal by day 4 and then returning to normal.

Mean platelet volume (MPV). Figure 2 shows that MPV measured in PRP anticoagulated with ACDE₁ was significantly increased for the first three days after APS treatment. On day 4 (96 hours) post-APS, when the rebound thrombocytosis was taking place, MPV was not significantly different from normal. The MPV correlated inversely with the degree of thrombocytopenia when the latter was expressed as a percentage of pretreatment platelet count ($P < .01$, $n = 25$, $r = .5560$, correlation coefficient and linear regression).

Morphology. Platelets in PRP (ACDE₁) were classified into different morphological forms by phase-contrast microscopy; their distribution is shown in Table 1. It is assumed that the nondiscoid platelets represent different morphological forms of proplatelets and their fragments. Pseudopod

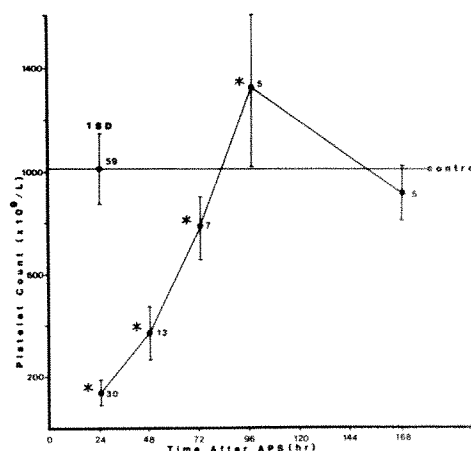


Fig 1. Platelet counts at intervals after anti-platelet serum (APS) injection. Numbers indicate number of rats per point. Values were significantly different from control: * $P < .001$.

formation and other evidence of platelet activation was minimal. Prefixation of blood drawn into syringes containing 0.1% glutaraldehyde did not significantly affect the morphology of platelets. Approximately equal proportions of proplatelets were present in IVC and aortic blood of both normal or APS-treated rats ($0.3 > P > .2$). The proportion of proplatelets in the venous and the aortic circulation was significantly increased 2 to 3 times at 24 hours after APS treatment (venous $P < .01$; aortic $P < .001$). In general, proplatelets produced at this time appeared to be longer, were more frequently beaded, and showed a larger number of beads per fragment than that seen in normal animals (Fig 3). All platelets collected into EDTA were spherical and did not display the morphology of proplatelets (Fig 4). This is presumed to be due to the spherizing effect of EDTA, although fragmentation of some very long proplatelets into smaller spherical forms cannot be excluded. Citrated platelets are apparently protected from the spherizing effect of EDTA, since their subsequent exposure up to a final concentration of 10 mg/mL of EDTA for 30 minutes did not change their morphology perceptibly. These circulating elongated nondis-

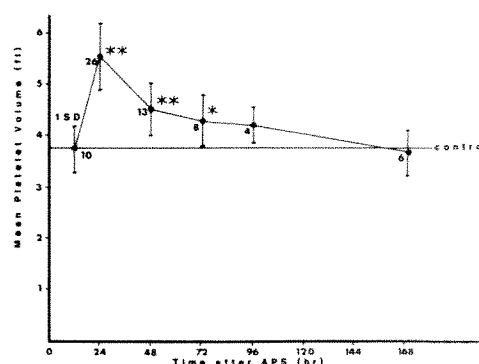


Fig 2. Mean platelet volume at intervals after anti-platelet serum (APS) injection. Numbers indicate number of rats per point. Data were collected from more than ten experiments. Values were significantly different from control: * $P < .05$, ** $P < .001$.

Table 1. Classification of Platelet Morphology by Phase-Contrast Microscopy in ACDE₁ Anticoagulant

Platelets	Normal, IVC (n = 6)	Normal, Aortic (n = 6)	24 Hours After APS* (IVC n = 6)	24 Hours After APS† (Aortic n = 7)
Discoid	93.86 ± 1.98	94.50 ± 2.95	81.75 ± 6.67	83.43 ± 3.41
Elongated	4.96 ± 1.51	3.60 ± 2.43	14.33 ± 4.52	10.57 ± 1.69
Beaded	0.63 ± 0.27	1.15 ± 0.59	2.10 ± 0.97	4.64 ± 2.64
String	0.7 ± 0.24	0.88 ± 0.77	1.87 ± 2.28	1.55 ± 0.39
Sum of nondiscoid forms (proplatelets)	6.28 ± 1.65	5.63 ± 3.00	18.30 ± 6.78	16.62 ± 3.45

ACDE₁, acid-citrated dextrose; IVC, inferior vena cava; APS, anti-platelet serum.

Percentage of platelets in platelet-rich plasma, mean ± SD.

At 24 hours after APS, the whole blood platelet counts were *9.5 ± 4.3% ($\bar{x}$ ± SD) and †8.0 ± 2.4% of the pretreatment platelet counts, respectively. There was no significant difference in the content of nondiscoid platelets between the above two blood circulations in either group of rats. The increase in beaded and string forms after APS treatment in either circulation did not reach statistical significance.

coid proplatelet forms are also readily demonstrable by transmission electron microscopy (Fig 5). Characteristic bundles of microtubules were seen, as described by Behnke¹⁸ in megakaryocyte pseudopodia and by Radley and Scurfield¹⁵ in proplatelets, running parallel to the long axis in the elongated proplatelets. Scanning electron microscopy also confirmed the presence of these elongated proplatelets among discoid platelets in the APS-treated rats (Fig 6).

DISCUSSION

It is now widely accepted that the megakaryocyte pseudopodia, first described by Wright¹⁹ in 1910 and subsequently recognized on phase contrast by Thiery and Bessis²⁰ and on electron microscopy by Behnke,¹⁸ actually penetrate the substance of endothelial cells to reach the marrow sinusoid^{14,15,21-23} and that platelets are not produced by direct

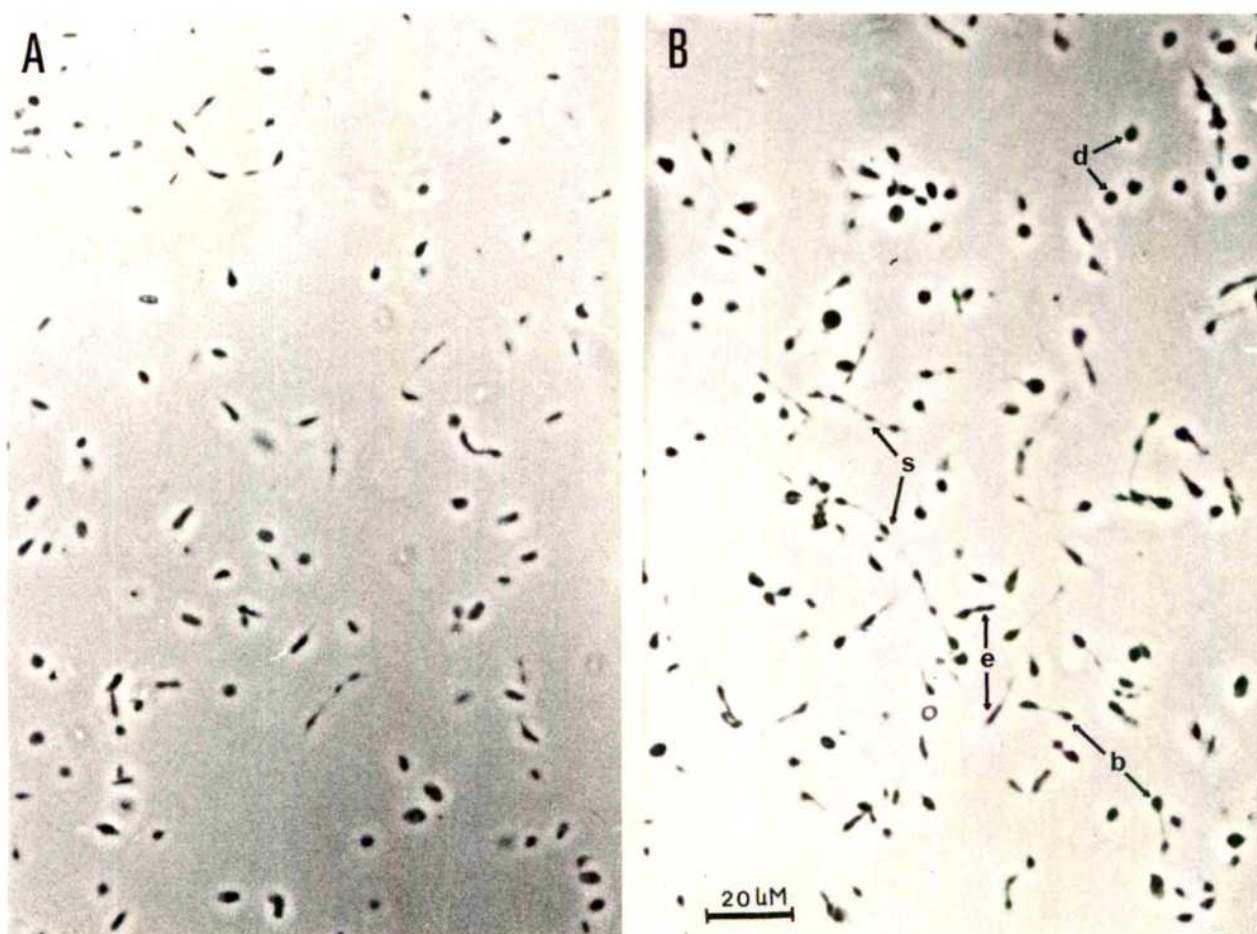


Fig 3. Phase-contrast micrographs of platelet-rich plasma anticoagulated with acid-citrated dextrose (ACDE₁) in (A) normal rats; and (B) in treated rats 24 hours after anti-platelet serum. In the latter, the numerous nondiscoid elongated forms and those showing constriction bands are collectively referred to as proplatelets. Examples of different platelet morphology are indicated: d, discoid platelet; e, elongated form; b, beaded form; s, string form.

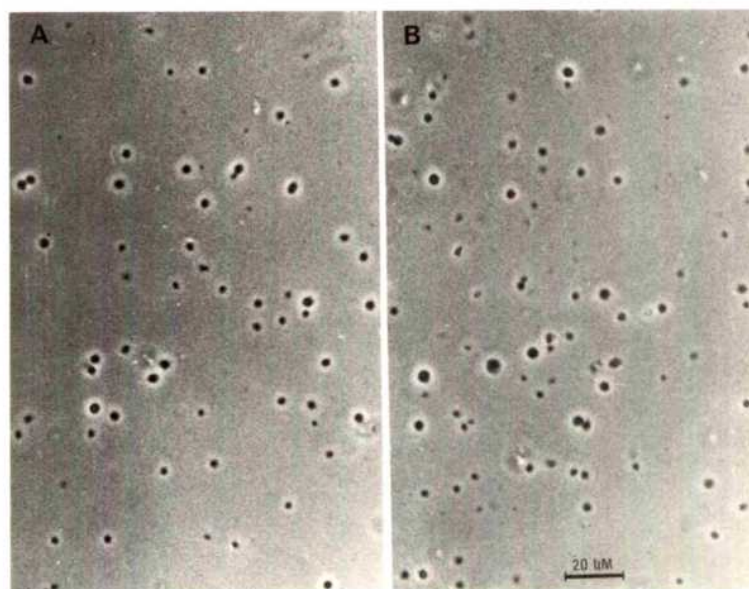


Fig 4. Phase-contrast micrography of platelet-rich plasma anticoagulated with EDTA in (A) normal rats; and (B) in treated rats 24 hours after anti-platelet serum. All platelets are spherical, and proplatelet forms are conspicuously absent.

shedding from megakaryocyte cytoplasm extravascularly within the bone marrow. The thrombopoietic megakaryocytes described by Paulus²⁴ in collagenase-digested marrow suspension are highly irregular in shape with multiple cytoplasmic extensions. By scanning electron microscopy, these long projections of megakaryocytes can be demonstrated in the marrow sinusoids^{14,15} and were termed proplatelets by Becker and de Bruyn.¹⁴ The parasinusoidal position of megakaryocytes in the bone marrow²⁵ and their extensive internal demarcation membrane system, thought to act as a reservoir of surface membrane,²⁶ are likely to facilitate this process of

pseudopod or proplatelet production. The actual mechanism of intraendothelial penetration and segment detachment in the sinusoid is not known. Speculation is focused at the possibility of cytoplasmic gel-sol transformation²⁷ made possible by the fluid nature of the demarcation membrane system through fusion-fission reorganization and its capacity for both evagination and invagination.²⁸

As the proplatelet processes first enter the sinusoids, they appear as villi containing longitudinally aligned microtubules and microfilaments.¹⁵ Similar pseudopod formation by megakaryocytes can be demonstrated in short-term *in vitro*

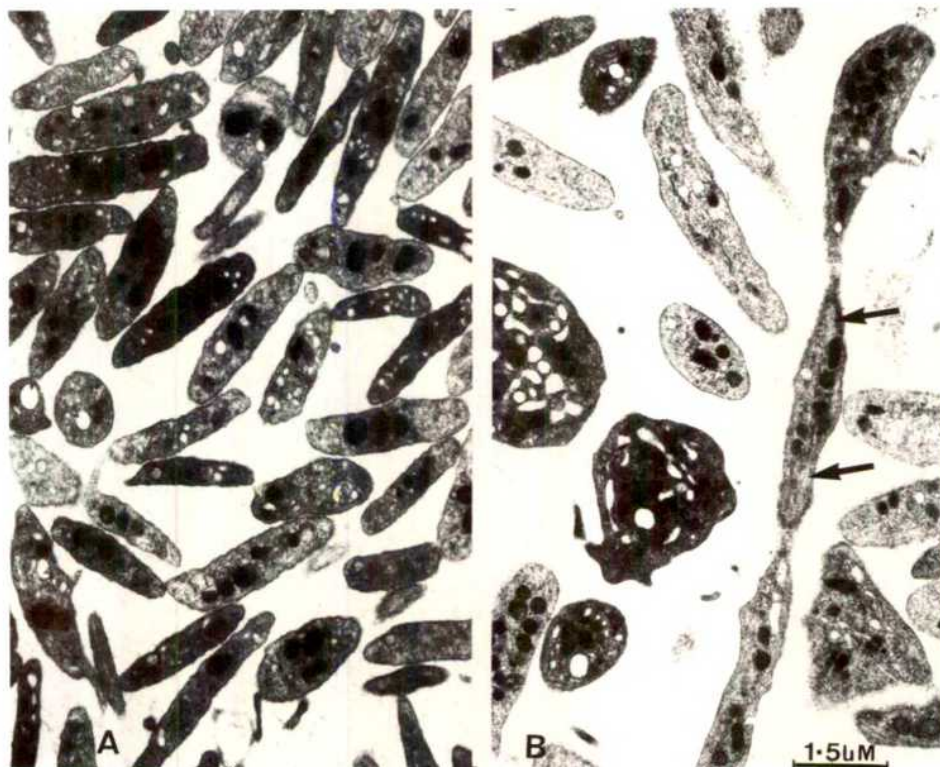


Fig 5. Transmission electron micrographs of acid-citrated dextrose (ACDE₁) anticoagulated platelet preparations in (A) normal rats; and (B) in treated rats 24 hours after anti-platelet serum. In the latter, a very long proplatelet with constriction bands is shown with its bundles of microtubules running longitudinally (↑).

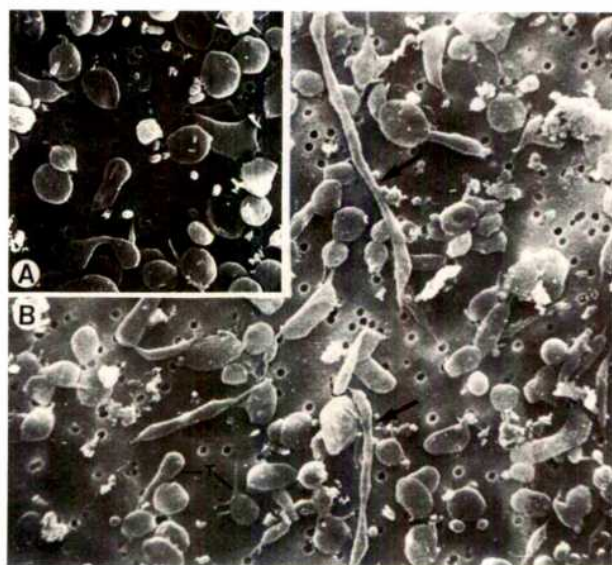


Fig 6. Scanning electron micrograph of platelets in (A) normal rats; and (B) in treated rats 24 hours after anti-platelet serum, showing the distinct contrast between discoid and elongated proplatelet forms ($\uparrow$). Other nondiscoid and tear drop forms (T) are also present (filter pore diameter 0.6 μ m).

cultures; by disassembling microtubules in vitro with vincristine or cold, the whole process of ribbon-like proplatelet formation can be reversed, indicating the importance of tubule structures in this process of proplatelet formation.²⁶ Recently, this process was recorded by time-lapse cinematography by Haller and Radley²⁹ on short-term culture of mouse megakaryocytes. The putative platelets in the chain of proplatelets exhibited some features common to circulating discoid platelets. These included flattening onto a glass surface and fine pseudopod formation comparable to that seen when platelets form. Rupture of the chains and release of platelets was not observed under in vitro conditions by these investigators, however, who postulated that individual platelets "bud off" from the proplatelets.

Using ACDE₁ as an anticoagulant, we described the presence of proplatelets in IVC and aortic blood of normal rats. These are not seen when EDTA is used as anticoagulant. Their features strongly resemble those of megakaryocytic proplatelet processes observed on scanning electron microscopy in marrow sinusoids. The circulating string forms represent proplatelet chains which would be expected to fragment further into shorter beaded forms. The smaller elongated forms may represent putative platelets that wait to be organized into discoid forms with the characteristic marginal microtubular bundles. During recovery from acute thrombocytopenia, the relative frequency of proplatelets and their nondiscoid fragments was increased threefold in the circulation; these included long segments with branches (Fig 7). Constrictions along the cytoplasmic processes are presumed to be the sites of future separation; hence, their distribution would dictate the platelet number and size to be produced. This hypothesis could be further tested by measurements of the distance between these constrictions in the

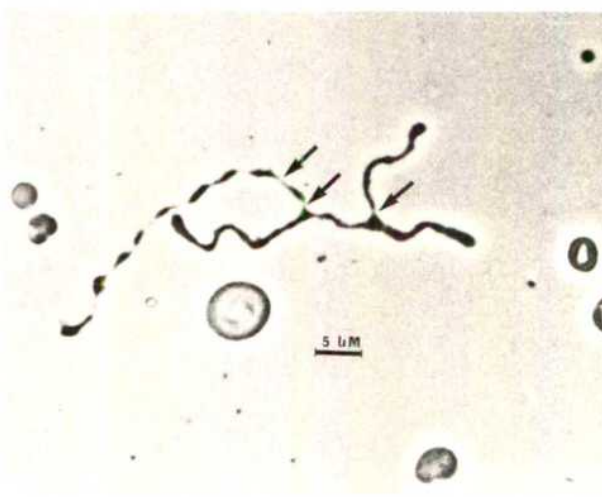


Fig 7. Phase-contrast micrograph of a branching proplatelet surrounded by a number of intact and fragmented RBCs together with various contaminant particles. What determines the number and location of the constrictions ($\downarrow$) in the proplatelet is not clear.

proplatelets of normal and acutely thrombocytopenic rats and comparison with the relative platelet volumes in these two situations. We propose that proplatelet chains break into fragments of lesser length at their narrowest, and presumably weakest, constriction sites rather than freeing individual platelets in a sequential manner from their distal ends. Such fragmentation may well occur within the microcirculation when proplatelets are caught astride bifurcating vessels. Subsequently, these circulating proplatelets must undergo internal reorganization into discoid platelets, presumably while "tumbling" in flowing blood or by some other mechanisms, because this change has not been observed in vitro.²⁹ Precisely how the lengthwise running microtubules of proplatelets rearrange themselves into the characteristic circumferential marginal microtubules of the discoid platelets remains to be determined.

Observations of circulating megakaryocytes³⁰ and pulmonary megakaryocytes³¹ both originating from the transmurular migration of whole megakaryocytes through the walls of the bone marrow sinusoid²¹ have been suggested as support for the notion of pulmonary platelet production.³²⁻³⁵ We failed to show any significant difference in proplatelet concentration between the venous and aortic circulation in both normal and APS-treated rats. The fact that bare nuclei of megakaryocytes are seen in bone marrow^{14,18} indicates that shedding of cytoplasm with production of proplatelets in marrow sinusoids must occur, as described by Becker and de Bruyn,¹⁴ and Scurfield and Radley.²² Our demonstration of free proplatelets in central venous circulation strongly reinforces this view. Twenty percent to 50% of mature megakaryocytes have been claimed to pass to the lung from the marrow.^{35,36} We assume that proplatelets are also released by these cells into the pulmonary venous circulation. The relative contribution of bone marrow and lung in platelet production has yet to be determined accurately.

This study demonstrated that beaded proplatelets representing fragments of megakaryocyte pseudopodia released

from marrow sinusoid can be recovered in the normal circulation. The proportion of these circulating proplatelets increased approximately threefold 24 hours after the induction of acute thrombocytopenia at a time prior to any significant increase in megakaryocyte size, ploidy, and number.^{7,8} During this recovery period, the proplatelets were visibly longer and more often beaded. These changes presumably resulted from accelerated proplatelet release from mature granular megakaryocytes in response to the thrombocytopenic stimulus. If proplatelet volume is four times greater than normal MPV, the increase in MPV observed at 24 hours could be entirely accounted for by the presence of >16% of proplatelets. The clear relationship between MPV and platelet count in the steady state observed by Levin and Bessman³⁷ and Giles³⁸ suggests, however, that fragmentation of proplatelets may give rise to discoid platelets of varying MPV depending on the circulating platelet count.

The morphology of these proplatelets is strikingly influenced by the anticoagulants used. Disassembly of their microtubules removes their elongated form even before release from megakaryocytes.²⁶ Like normal platelets, they are spheroid by EDTA (Fig 4). Macrothrombocytes appearing in idiopathic thrombocytopenic purpura (ITP) or other clinical conditions associated with altered platelet production such as myeloproliferative disorders may include a significant number of proplatelets spheroid by EDTA prior to preparation of blood smears. The megathrombocyte of the peripheral blood film³⁹ therefore may be either a spheroid "young" proplatelet or the "large" end of the log-normal distribution.

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Antibodies to Granulocyte Precursors in Selective Myeloid Hypoplasia and Other Suspected Autoimmune Neutropenias: Use of HL-60 Cells as Targets

By Mark S. Currie, J. Brice Weinberg, Pradip K. Rustagi, and Gerald L. Logue

Patients with syndromes of autoantibody-mediated hemacytopenias may manifest signs of increased cell destruction and/or decreased cell production, depending on the maturity of the target cell and the effects of antibody binding. The purpose of this study was to use a cultured human cell line of hematopoietic origin for in vitro assays of antibody binding to overcome the relative inaccessibility of natural human marrow progenitor cells. This report describes the detection, using radioiodinated staphylococcal protein A (SPA), of antibodies binding to a human promyelocytic cell line (HL-60) in sera from three patients with chronic idiopathic granulocytic hypoplasia ("pure white cell aplasia," PWCA) and 22 patients with other syndromes of suspected immune neutropenia. Bone marrow from patients with increased IgG binding to HL-60 cells showed <15% granulocytic lineage cellularity in 11 of 17 cases. In vitro differentiation of HL-60 cells by retinoic acid resulted in increased IgG binding for sera that had shown

increased IgG binding to mature granulocytes but not undifferentiated HL-60 cells; in contrast, for sera with antibodies to untreated HL-60 cells and for normal serum, in vitro differentiation had little effect on IgG binding. Antibodies eluted from mature granulocytes were similar to the parent serum regarding the ratio of IgG binding to mature cells v HL-60 cells. No sera from 19 patients with febrile transfusion reactions showed increased IgG binding to HL-60 cells in the absence of increased IgG binding to mature granulocytes, although two sera had antibodies to both cell types. The use of HL-60 cells as targets may permit measurement of serum antibodies associated with granulocytic hypoplasia. In combination with assays to detect antibody binding to mature granulocytes, these techniques may discriminate among autoantibody specificities for antigens that are gained, conserved, or lost during myeloid maturation.

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ANTIBODIES that bind to mature blood elements and their marrow progenitors have been detected in immune cytopenias affecting RBCs, platelets and neutrophils. Although assays for antibody binding to peripheral blood cells have been available for many years,¹ the detection of serum antibodies to early marrow precursors has depended primarily on use of in vitro clonogenic assays of marrow progenitor cells.² In the present study, we demonstrate the use of a human myeloid cell line (HL-60) to detect antibodies to granulocyte precursors in syndromes of immune neutropenia. The pathophysiologic significance of antibodies so detected is implied, in part, by clinical correlation of their presence with bone marrow myeloid hypoplasia.

MATERIALS AND METHODS

Cases. Patient 1 is a 69-year-old white man first noted to be neutropenic in early 1982. All hematologic values and serum chemistries were in the normal range except for the granulocyte count (300/ μ L). Assays for rheumatoid factor, antinuclear antibodies, and VDRL were negative. Bone marrow biopsies show a cellularity of 20% with erythroid predominance (M/E ratio of 1:4) and no mature granulocytes. Wright's stain of the marrow aspirate showed no granulocyte precursors beyond the late promyelocyte or early myelocyte stage. Average hematologic values from 1982 through 1985 are shown in Table 1. Physical exam showed no organomegaly and was remarkable only for ulatrophia. The patient has remained in excellent health except for one hospitalization, during which intravenous (IV) antibiotics were administered for an acute febrile episode without identified source of infection. He has also received penicillin VK (3 to 4 days every 2 to 6 months) for aphthous mouth ulcers or fever without identified source of infection. He takes no other medications and has no complaints other than occasional fatigue, and no other significant health problems have been identified.

Patient 2 is a 66-year-old black man who has been followed for neutropenia since 1982. Normal complete blood counts were documented in 1974 and 1976. In 1977, his hemoglobin, hematocrit, and platelet count were normal, but there were 1,200 granulocytes/ μ L and, in early 1982, 650 granulocytes and 100 monocytes/ μ L were recorded. Over the past 2 years, mean absolute granulocyte count has been 210/ μ L (Table 1). Radionuclide scan of the liver and

spleen was normal. Flow cytometric immunofluorescence analysis of peripheral blood lymphocytes to assess markers for B cells, helper, suppressor and total T cells, and natural killer cells showed no abnormalities. Serum protein electrophoresis showed a polyclonal increase in γ globulins to 20 mg/mL. At a 1:160 dilution, antinuclear antibodies with a speckled pattern were detected by indirect immunofluorescence using HEP-2 cell monolayer as substrate. Neither rheumatoid factors nor anti-DNA antibodies were detected. Bone marrow biopsies demonstrated a cellularity of 20% to 30% with erythroid predominance (M/E ratio of 1:10). Rare granulocytic cells beyond the myelocyte stage were present, and there were focal lymphoid aggregates with benign morphology. Bone marrow biopsies in 1982 and 1983 were both interpreted as "marked granulocytic hypoplasia." Complete blood counts on three first-degree and two

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Table 1. Mean Hematologic Values for Three Patients With Selective Granulocytic Hypoplasia

Parameters	Total Leukocytes*	Neutrophils*	Monocytes*	Lymphocytes*	Platelets*	Hemoglobin†
Patient 1	2,600	230	560	1,600	219,000	14.4
Patient 2	1,200	210	140	780	218,000	10.6
Patient 3	2,800	900	600	1,200	182,000	12.7

Mean values were calculated from the results of 10 to 12 complete blood counts obtained on each patient at one- to six-month intervals between January 1982 and January 1986.

*Results are expressed as cells per microliter of whole blood.

†Hemoglobin is expressed as g/100 mL of whole blood.

second-degree relatives showed each to have a WBC count $>5,000/\mu\text{L}$. The patient was admitted to the Durham VA in March 1985 with fever, pain, and erythema of the right flank. Despite prompt institution of antibiotics appropriate to the *Staphylococcus aureus* subsequently cultured, the area progressed to abscess formation requiring surgical drainage. The patient has been on various β -blocker drugs since 1974 for idiopathic hypertrophic subaortic stenosis. He has also been treated at different times with digitoxin, quinidine, hydrochlorothiazide, and nitrates, but neither institution nor discontinuation of any of these drugs has been associated with a change in neutrophil counts.

Patient 3 is a 61-year-old black man followed since 1972 for mild depressive reactions and varicose leg veins. A normal WBC count was documented in 1957. In mid-1982, routine blood counts revealed neutropenia and he was referred for further evaluation. At that time, his WBC count was $2,500/\mu\text{L}$, of which 600 were neutrophilic granulocytes. His complete blood counts have remained remarkably stable, with mean hemoglobin of 12.7 g/dL, WBC count of $2,800/\mu\text{L}$, granulocytes $900/\mu\text{L}$, and monocytes $600/\mu\text{L}$ (Table 1). Bone marrow biopsy showed a cellularity of $\sim 30\%$ with erythroid predominance (M/E ratio of 1:4) with few mature granulocytes. He has been on no medications for several years and has suffered no unusual infections. None of the three patients has been transfused.

Buffers. The buffers used were: Veronal-buffered saline, pH 7.5 (VBS); VBS with 0.15 mmol/L of Ca^{2+} and 0.5 mmol/L of Mg^{2+} (VBSE); VBS with 0.1 mmol/L of EDTA (VBSE); VBS with 5% fetal bovine serum (FBS), (VBSF); Alsever's (acid-citrate-dextrose); Alsever's with 5% dextran (ACDD); phosphate-buffered isotonic saline with 2% paraformaldehyde (PFS); and VBS made 20% dimethylsulfoxide (VBSD).

Cells. Granulocytes were obtained from healthy volunteers with group O blood type. Venous blood was drawn into a heparinized syringe (10 U/mL of blood), diluted 9:1 with ACDD, and sedimented at 1 g for 1 hour at ambient temperature (RT). Granulocytes were separated from RBCs, lymphocytes, platelets, and plasma by centrifugation through a double layer of Ficoll-sodium diatrizoate mixtures with specific gravities of 1.077 and 1.120 as previously described.³ Granulocytes were collected from the 1.077/1.120 interface, washed twice in ACD, resuspended in ACD, and mixed with an equal volume of PFS. After incubation for 10 minutes at RT, the paraformaldehyde-fixed granulocytes (PFGs) were washed twice in VBS, resuspended in VBSD, and frozen at -70°C . Immediately before use, PFGs were thawed, washed in VBSE, and adjusted to a concentration of 20 to $30 \times 10^6/\text{mL}$. All washing was by centrifugation in 10 mL of buffer at 300 g for 10 minutes at RT. Thawed PFG preparations demonstrated $>95\%$ intact granulocyte morphology on Wright's-stained preparations. The HL-60 (human promyelocytic leukemia) cell line was from the American Tissue Culture collection. The cells used in differentiation experiments were from passage numbers 28 through 40. Cells were passed 2 to 3 times a week in RPMI 1640 with 10% FBS at 2 to 4×10^5 cells/mL. Cells used in initial IgG binding assays were harvested during log-phase growth,

washed in ACD, fixed, stored, and assayed like PFG. Freshly fixed HL-60 cells that had not been frozen were used for complement fixation assays. Leukemic myeloblasts and promyelocytes were obtained from the WBC-rich plasma of a patient with acute myelogenous leukemia, with a total WBC count $>100,000/\mu\text{L}$, of which 50% were promyelocytes and 50% were myeloblasts by Wright's stain morphology consistent with French-American-British (FAB) classification M2. These cells were also fixed, stored, and assayed in the same manner.

To study the effect of differentiation on IgG binding, cells were grown for 5 to 7 days concurrently under three conditions⁴ (a) in RPMI 1640 with 10% FBS alone (control), (b) in the same media with 1 $\mu\text{mol/L}$ of 13-*cis*-retinoic acid, or (c) with 12% (wt/vol) dimethylsulfoxide. Cells were harvested, counted, assayed for viability by trypan blue exclusion, and assayed for capacity to reduce nitroblue tetrazolium in the presence of 200 nmol/L of phorbol myristate acetate,⁴ or washed in ACD, fixed in paraformaldehyde, stored, and assayed in the same manner as PFGs and log-phase HL-60 cells. Granulocytes and log-phase HL-60 cells routinely demonstrated $>90\%$ viability prior to fixation. Viability was as low as 72% for cells cultured 7 days but was unaffected by the presence or absence of retinoic acid or dimethylsulfoxide (DMSO).

Sera. Sera were obtained, conforming to policies of Duke and Durham VA human subjects committees from normal donors, multiply transfused patients, and patients with suspected immune neutropenia (granulocytes $<2,000/\mu\text{L}$). Whole blood was clotted at room temperature, and the serum was removed and divided into 0.3-mL aliquots that were frozen at -70°C . For 1 year, ~ 200 sera were tested for evidence of antigranulocyte antibodies. All were tested for IgG binding to PFG as described below, and approximately one-fourth were also tested for IgG binding to HL-60 cells either because tests of IgG or IgM binding or C3 fixation to PFG were positive or because immune etiology was strongly suspected and/or clinical information (eg, chronicity, bone marrow findings) was sufficient to warrant further study. The patients' sera reported here are those that were "positive" (defined as more than two times normal IgG binding to PFG or HL-60 cells), and for which clinical information regarding drug use and associated disorders was known.

Eluates. PFG sensitized in undiluted serum were washed three times in VBS and resuspended in VBSE, and bound antibody was eluted in a fivefold volume of 1 mol/L of NaCl, 0.1 mol/L of citric acid pH 2.4 for 10 minutes at 4°C . After centrifugation at 2,000 g for 1 minute, the supernatant was removed and neutralized to pH 7.2 with 0.1 mol/L of NaOH. The eluate was then diluted to isotonicity (ratio of starting serum to final eluate volume was 1:20).⁵

Antibody testing. Radioiodinated SPA was used to quantitate granulocyte binding IgG.^{3,6,7} The target cells were incubated in a dilution of test serum (1:20 in VBSE unless otherwise specified) for 20 minutes at RT followed by 10 minutes at 37°C . After incubation, the cells were washed three times in VBSF and resuspended in 0.2 mL of VBSF, and 0.05-mL aliquots were incubated in triplicate with

5 ng of radioiodinated SPA (labeled by Bolton Hunter reagent to specific activity of 30 to 40 mCi/mg by Amersham) in 0.05 mL of VBS for 30 minutes at RT, layered over 0.1 mL of a 6:5 mixture of *n*-butyl and *bis*-hexyl phthalate oils. The remaining cell suspension was reserved for duplicate cell counts using a Coulter DN2. The cells were washed by centrifugation through the oil in a Beckman Microfuge B for 3 minutes, thus separating cell-bound from unbound SPA. The microfuge tubes were clamped beneath the aqueous oil interface, the tips were cut off, and the γ emissions were measured in a Beckman Gamma 4000. IgG bound is expressed for purposes of comparison as femtograms (fg) of SPA per cell. IgM binding was measured in the same way, except that radioiodinated monoclonal anti-human IgM (Boehringer-Mannheim) at 3 μ g/mL was substituted for SPA and 25 μ g of IgG (purified by ion-exchange chromatography from the serum of a patient with IgG multiple myeloma) was included to lower background binding of the radiolabeled antibody. The ability of sera to cause C3 fixation to PFG and HL-60 cells was measured in an assay similar to one we previously described.³ In this assay, the cells are incubated with test serum diluted 1:20 in VBSM for 15 minutes at RT, washed, and then resuspended in freshly thawed type AB serum at a final dilution of 1:10 in VBSM. The 37 °C incubation, washing, and radioligand binding steps are otherwise the same, except that radioiodinated monoclonal antihuman C3c (Bethesda Research Laboratories) at 6 μ g/mL is used for the final incubation. Assays of IgG and IgM binding to the washed cells that had been incubated with test serum plus the 2.5-fold excess of normal serum used as complement source gave very similar results to those for cells incubated in test serum alone. This suggests that increased antibody binding was due to specific antibody in test sera and that increased amounts of nonspecific antibody in the initial incubation did not affect results for this assay system. Other cell types including RBCs, lymphocytes, platelets, and paraformaldehyde-fixed blasts and promyelocytes (50% each) from a patient with acute myelogenous leukemia (FAB classification M2) were substituted in the above assays in some experiments. All cells except RBCs had been fixed in 1% paraformaldehyde for the assays reported.

RESULTS

Sera from the three described patients with selective myeloid hypoplasia bound significantly more IgG to HL-60 cells than did control sera ($P < 0.01$), whereas IgG binding to PFGs was not significantly different, ($P > .1$; Table 2). Thus, these neutropenic patients who did not have detectable serum antibodies to mature granulocytes, did have antibod-

ies to an immature myeloid cell associated with a marked reduction of bone marrow granulocyte precursors.

When sera from other patients with suspected immune neutropenia were tested, >20 had increased IgG binding to either HL-60 cells alone or to both HL-60 cells and mature granulocytes. IgG binding to PFG and HL-60 cells, quantitated as fg SPA/cell is plotted for normal and positive patient sera in Fig 1. A positive result was arbitrarily defined as greater than two times the mean value for normal serum. As depicted in Fig 1, patient groups could be divided into those whose sera showed increased IgG binding to PFG only (group I), both PFG and HL-60 cells (group II), or HL-60 cells only, as found for the three patients above (group III). Table 3 lists the bone marrow findings and the major associated drug or disease for the patients whose antibody testing results are plotted in Fig 1. Bone marrows from group I patients showed a normal or increased number of granulocytic cells, whereas those of group III patients had decreased myeloid cellularity. Findings were variable in group II patients, with some showing hyperplasia and some showing marked hypoplasia of the myeloid series. Consistency of findings associated with a given drug or disease could not be assessed in neutropenic conditions for which only one or two positive sera were identified. Four or more sera were identified, however, for patients with neutropenia associated with procainamide treatment, rheumatoid arthritis, and large granular lymphocytosis (LGL) (defined by the persistent

Table 2. IgG Binding to Granulocytes and HL-60 Cells for Normal and PWCA Sera

Sera	fg SPA/Cell	
	PFGs	HL-60
Normal (n = 9)	0.32 $\pm$ 0.10	0.24 $\pm$ 0.09
Patient 1	0.26 $\pm$ 0.08	0.75 $\pm$ 0.11
Patient 2	0.34 $\pm$ 0.14	0.69 $\pm$ 0.09
Patient 3	0.44 $\pm$ 0.06	0.93 $\pm$ 0.15

PWCA, pure white cell aplasia; SPA, staphylococcal protein A; PFGs, paraformaldehyde-fixed-granulocytes.

Patient sera were tested for IgG binding to each cell type on at least three occasions as described. Normal sera were included in each assay. The mean $\pm$ SD femtograms of SPA bound per cell is given for each patient and the group of nine normal sera. For HL-60 cells, patient results are significantly greater than normal ($P < .01$ by Student's *t* test).

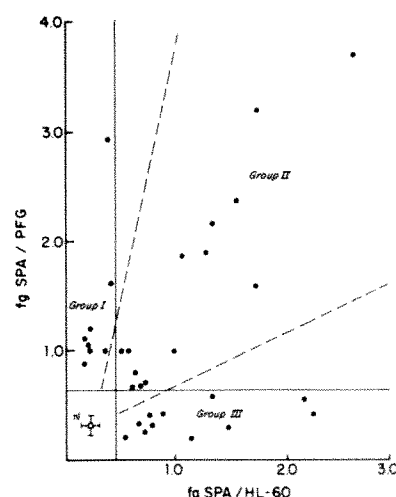


Fig 1. IgG binding to mature granulocytes v HL-60 cells. Solid circles: IgG binding (as measured by mean staphylococcal protein A (SPA) bound per washed cell in two or more triplicate assays) to paraformaldehyde-fixed granulocytes (PFGs) and HL-60 for 33 individual sera tested at a 1:20 dilution. The mean and SD for nine assays of normal sera tested concurrently are depicted by the open square with vertical and horizontal brackets. Positive results for patient sera arbitrarily defined and grouped by values exceeding twice the normal mean for SPA binding to PFGs and/or HL-60 are indicated by the horizontal and vertical solid lines, respectively. Dashed lines drawn from the open square reflect a similar division of groups of sera according to the ratio of excess IgG binding to PFGs v HL-60. For clarity, data for 140 other patient sera that resulted in less than twice the mean SPA binding for normal subjects are not shown.

Table 3. Patients With Increased Antibodies to Mature Granulocytes and/or HL-60 Cells

Patient Designation	Association*	fg SPA/Cell† PFG/HL-60	IgM‡ PFG/HL60	C3‡ PFG/HL60	Age/Sex	Cellularity§
Group I						
4	C	1.0/0.3	-/-	+/-	54 M	↑
5	AMT	0.9/0.2	-/-	+/-	29 F	I
6	LGL	1.2/0.2	-/-	-/-	71 F	I
7	LGL	1.1/0.2	-/-	-/+	56 F	↑
8	RA	1.1/0.2	-/-	+/+	54 M	↑
9	RA	1.0/0.4	-/-	+/+	55 M	↑
10	RA	1.6/0.4	-/-	+/+	63 M	↑
11	RA	2.9/0.4	-/-	+/+	67 M	↑
Group II						
12	RA	3.2/1.8	+/+	+/+	67 M	↑
13	RA	2.2/1.4	+/-	+/+	59 M	I
14	RA	1.0/1.0	+/+	+/+	64 F	I
15	RA	0.8/0.7	+/-	-/+	65 F	I
16	RA	1.0/0.5	-/-	+/+	58 F	—
17	RA	2.4/1.6	-/-	+/+	40 M	↑
18	P	1.6/1.8	-/-	+/+	65 F	↓
19	P	3.7/2.7	+/+	+/+	81 F	—
20	P	1.9/1.1	-/-	+/+	71 F	↓
21	P	0.7/0.7	-/-	+/+	65 M	↓
22	HT	1.9/1.3	+/+	+/+	60 F	—
23	HCL	0.7/0.7	-/-	+/+	56 M	—
24	LGL	1.0/0.6	-/-	+/+	54 M	—
25	LGL	0.7/0.6	-/-	+/+	53 F	↓
Group III						
26	LGL	0.2/1.2	-/-	-/-	54 M	↓
27	HD	0.4/2.3	-/-	-/-	37 M	↓
28	IM	0.3/0.8	-/-	-/-	17 F	I
29	AA	0.2/0.6	-/-	+/+	57 M	↓
30	AA	0.4/0.8	-/-	-/+	76 F	↓
31	SS	0.6/2.2	-/-	-/+	49 F	—
32	LS	0.6/1.4	+/+	+/+	55 M	—
33	CC	0.3/1.5	+/+	+/+	70 F	—
1	PWCA	0.3/0.8	-/-	+/+	69 M	↓
2	PWCA	0.3/0.7	-/-	+/+	66 M	↓
3	PWCA	0.4/0.9	-/-	+/-	60 M	↓

All patients had <2,000 granulocytes/ μ L when serum was obtained.

*The associated drugs or diseases were: C, cephalosporin; AMT, autologous marrow transplantation; LGL, large granular lymphocytosis; RA, rheumatoid arthritis; P, procainamide; HT, hyperthyroidism; HCL, hairy cell leukemia; HD, Hodgkin's disease; IM, infectious mononucleosis; AA, acute aplastic anemia; SS, "serum sickness"; LS, latent syphilis; CC, cryptogenic cirrhosis; and PWCA, chronic idiopathic myeloid hypoplasia or pure white cell aplasia; other abbreviations as in Table 2.

†Mean value of two or more assays is recorded. In a typical assay, the mean SE of triplicate results was 5%, with a maximum of 10%. Results for the same patient assayed on different days varied as much as 40%, but the SEM of three or more assays was usually <20%. For nine normal sera tested, the mean SPA binding was 0.32 ± 0.11 fg/PFG and 0.26 ± 0.10 fg/HL-60 cell.

‡Results for anti-IgM and anti C3c binding are recorded as positive (+) if ≥ 1.4 times the normal mean and as negative (-) if <1.4 times the normal mean.

§Myeloid cellularity was calculated from the percentage of overall hematopoietic cellularity and the myeloid/erythroid ratio in the official pathology reports if available. Myeloid cellularity is designated as decreased (↓) if <15%, as intermediate (I) if between 15% and 40%, and as increased (↑) if >40%. We confirmed results for 14 marrows available for review.

presence of >500/ μ L lymphoid cells with azurophilic granules on Wright's stain and/or HNK-1 monoclonal antibody binding on flow-cytometric immunofluorescence analysis of peripheral blood mononuclear cells). As shown in Table 3, observations based on these clinical associations were as follows. Sera from patients with procainamide-related agranulocytosis were in group II, demonstrating increased IgG binding and C3 fixation to both PFG and HL-60 cells. Patients with Felty's syndrome fell into both group I and group II, but all had PFG/HL-60 IgG binding ratios of ≥ 1

and their bone marrows did not show myeloid hypoplasia. Patients with LGL and neutropenia were noted in all three groups. Bone marrow biopsies were available from three patients with LGL (patient 6 in group I, patient 25 in group II, and patient 26 in group III). Marrow from patient 26 showed myeloid hypoplasia, whereas marrow from patient 6 was normocellular with a selective decrease in mature granulocytes. Marrow from patient 25 was 80% cellular with 65% replacement by abnormal lymphocytes (as compared with <10% lymphs for patients 6 and 26). The remaining patients

represent a variety of conditions in which immune neutropenia or other autoimmune phenomena have been described, as detailed in Table 3.

The different PFG/HL-60 IgG binding ratios that divide patients into groups demonstrating antibodies binding primarily to mature granulocytes (group I), immature myeloid cells (group III), or both (group II), might each be the result of single or combined antibody specificities for antigens that are variably expressed depending on the degree of myeloid maturation. To address the question of whether sera contained antigranulocyte antibodies to one or more than one antigen, several eluates were prepared from PFGs incubated in sera with different PFG/HL-60 IgG binding ratios. If a serum contained antibodies with multiple specificities differing in PFG/HL-60 binding ratio, one might expect the PFG eluate to show a higher PFG/HL-60 binding ratio than the original serum by virtue of a greater representation of predominantly PFG binding antibody in the PFG eluate. Alternatively, if a serum contained antibodies of a single specificity, so that the PFG/HL-60 binding ratio was determined by the ratio of antigen available on mature v immature cells, the PFG eluate and its parent serum should demonstrate the same PFG/HL-60 binding ratio. In Fig 2, the PFG/HL-60 binding ratio of serum is plotted against the ratio for the corresponding eluate assayed simultaneously. For the six sera tested, points fall on a line close to unit slope (0.84), consistent with a single specificity in each serum.

If the different PFG/HL-60 IgG binding ratios are due to different antibody specificities for antigens that are gained, conserved, or lost during neutrophil maturation, one might expect to see changes in antibody binding to HL-60 cells after in vitro differentiation. As illustrated in Fig 3, sera from group I patients showed an increase in IgG binding to HL-60 cells grown in the presence of retinoic acid or

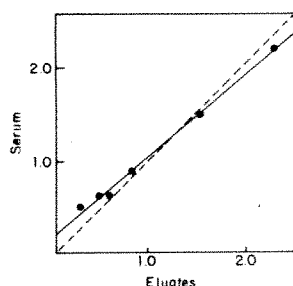


Fig 2. Paraformaldehyde-fixed granulocyte (PFG)/HL-60 IgG binding ratios for serum v PFG eluates. For six sera (from patients 3, 11, 13, 17, 18, and 20) and the six eluates prepared from PFGs incubated in these sera, IgG binding to PFG and HL-60 cells was assayed simultaneously and the ratio of staphylococcal protein A (SPA) binding to each cell type was calculated. The ratios for each patient's serum and corresponding PFG eluate provide the coordinates for the six solid circles. Each falls close to a line with slope of 0.84, which intersects a line from the origin with unit slope at (1.3, 1.3). Unit slope is represented by the dashed line where points would be expected to fall if binding ratios were the same for eluate and serum. More selective PFG binding by eluates would cause points to fall to the right of the dashed line. The direction of deviation from unit slope and point of intersection may be explained by the effects of nonspecific IgG binding in whole serum, since normal sera give a mean PFG/HL-60 SPA binding ratio of 1.2.

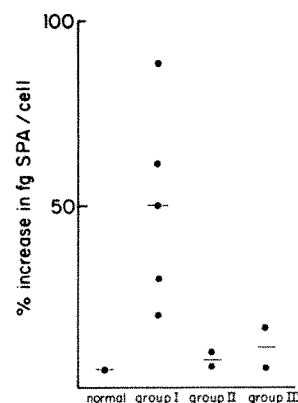


Fig 3. Data from a representative experiment measuring the effect of differentiation on IgG binding to HL-60 cells. Cells were cultured for 7 days in the presence or absence of $1 \mu\text{mol/L}$ of 13-*cis*-retinoic acid (RA) and fixed. The percentage of increase in staphylococcal protein A (SPA) bound to retinoic acid-treated cells as compared with control HL-60 cells is plotted for normal serum and sera from patients in each of the previously defined groups. In this experiment, 22% of control HL-60 cells reduced nitroblue tetrazolium (NBT), whereas 61% of the RA-treated cells did. Similar results (not shown) were obtained in replicate experiments and also when cells were cultured in the presence of 1.2% dimethylsulfoxide (DMSO).

dimethylsulfoxide (DMSO). Essentially no change was seen for sera from patients in group II and group III. The HL-60 cell line may spontaneously lose the capacity to differentiate in response to these agents over time.⁸ Retinoic acid treatment of such HL-60 cells that have lost the capacity to differentiate (as assessed by morphology, nitroblue tetrazolium reduction, and anti-CR1 binding before and after 7 days of DMSO or retinoic acid treatment) in the course of prolonged continuous culture (>300 passages) failed to show any change in the amount of IgG bound by sera from group I patients. Thus, antibodies in sera from group I patients appear to bind to antigens acquired during differentiation.

The data for C3 fixation (Table 3) also suggest that mature granulocytes and HL-60 cells share antigens that are targets in immune neutropenia. Of 29 sera causing excess C3 fixation to one cell type, 22 (76%) caused excess C3 fixation to the other cell type as well, regardless of whether the ratio of IgG binding favored mature granulocytes (as with sera from neutropenic patients with rheumatoid arthritis) or HL-60 cells (as with sera from the myeloid hypoplasia patients). In contrast, $<2\%$ of sera tested have been found to cause excess C3 fixation to granulocytes in the absence of increased antibody binding to either PFG or HL-60 cells. Of the 29 sera reported here to cause at least 40% more than normal C3 fixation to PFG or HL-60 cells, we found only increased IgG binding and not IgM binding in 21, and increased binding of both immunoglobulin classes in 8. We have not identified sera that resulted in increased binding of anti-IgM to sensitized granulocytes at 37°C without also causing increased SPA binding. Thus, the potential importance of complement-fixing IgM antigranulocyte antibodies remains to be determined.

To demonstrate selective myeloid specificity of antibodies binding to HL-60 cells, sera from the three patients with

PWCA were tested for IgG antibody binding to RBCs, platelets, and lymphocytes from a normal group O donor and to the blasts and promyelocytes from a patient with acute myelogenous leukemia. Serum IgG binding to RBCs, lymphocytes, and platelets was no different for the three PWCA patients as compared with normal control subjects. As Fig 4 shows, however, the sera from these three patients did show increased binding to freshly isolated acute myelogenous leukemia cells as well as to the in vitro-passaged HL-60 cells.

In data not shown, sera from two groups of multiply transfused patients were tested for IgG binding to HL-60 cells. The first group of sera were from nine patients who had received RBCs or platelets from ten or more donors because of cytopenias related to chemotherapy or marrow disorders. None of these sera were positive as previously defined. The second group of sera were obtained 2 to 5 days following report of a febrile transfusion reaction in 30 patients. Two sera bound more than twice as much IgG to HL-60 cells as compared with normal serum. Both these sera also bound more than twice normal amounts of IgG to PFGs. Thus, allosensitization resulting in antibodies that bind to HL-60 cells but not mature granulocytes was not observed.

Fifty sera from healthy donors aged 64 to 89 years (median 81) were also tested, and mean ($\pm$ SD) for SPA binding of 0.34 ($\pm$ 0.14) fg/PFG and 0.23 ($\pm$ 0.08) fg/HL-60 were not different from younger controls (Table 2). Values greater than twice normal were found for only 1 of these 50 sera, and this one sample caused increased IgG binding to both PFG and HL-60 cells.

DISCUSSION

One expects to see compensatory hyperplasia of marrow precursors in processes that cause cytopenia by an effect on the postmitotic, terminally differentiating cells. An apparent reduction in the proliferating pool of committed precursors implies, however, that the corresponding cytopenia is due to effects on the more immature hematopoietic progenitors. Although hematologic malignancies, infections, inflammatory processes, and bone marrow infiltrations may preferentially reduce one cell line more than others, the isolated

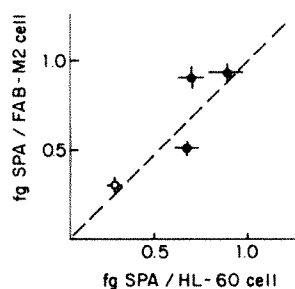


Fig 4. Results for triplicate assays of IgG binding to freshly isolated and fixed acute myelogenous leukemia, French-American-British classification M2 cells as compared with the HL-60 cells are shown for normal serum (open circles) and three patients with pure white cell aplasia (PWCA) (solid circles), with SE brackets for each value in femtograms of staphylococcal protein A per cell. The dashed line indicates unit slope.

reduction in a single cell line should raise the suspicion of an autoimmune process.

Suspected immune cytopenias with precursor cell hypoplasia have been associated with both serum (IgG $\pm$ complement) and cell-mediated (T lymphocyte) processes in studies based on in vitro clonogenic assays.⁹⁻¹⁹ Also, clinical responses to plasmapheresis²⁰ and antilymphocyte globulin treatments²¹ have suggested roles for antibody and/or effector cells in mediation of these disorders, but the detailed pathophysiology remains a matter of inference and speculation. In this study, we found evidence of antibodies specific for immature myeloid cells in a number of patients with apparent granulocytic hypoplasia. Three cases demonstrated acquired chronic hypoplasia of the granulocytic series. Although patient 2 had a mild reduction in RBCs, hypergammaglobulinemia, and a low-titer antinuclear antibody, these patients otherwise had no generalized hematologic disorders or associated systemic diseases. Because the PWCA patients were untransfused males, allosensitization could not explain the increased antibody binding to the HL-60 or FAB-M2 leukemic cells, and it is not a likely explanation for the findings in other patients in group III. The term "pure white-cell aplasia" as it has been applied to idiopathic, disease-associated, or drug-induced hypoplasia of the granulocyte series,¹⁶⁻¹⁸ is a misnomer since nongranulocytic leukocytes may be unaffected, and the variably severe reduction in the proliferative pool of granulocyte precursors reflects degrees of hypoplasia rather than aplasia. The term seems appropriate, however, in its implication of the analogy to "pure red cell aplasia" as applied to immunologically mediated hypoplasia of the erythroid series.⁹

It is difficult to prove that antibodies that bind to cells are the cause of syndromes such as blood cell cytopenias, particularly granulocytopenia. Both granulocyte production and the distribution of cells between the marrow, the marginal and circulating blood pools, and the tissues are subject to many variables. These factors make studies of antibody effects on granulocyte survival and kinetics more difficult to perform and interpret than corresponding studies on RBCs and platelets.²² Effects occurring in the bone marrow are even less accessible to observation. For the granulocyte, which is $\sim$ 10 days in production as compared with perhaps 10 hours in the circulation, immune damage in the marrow may be of central importance regardless of the maturity of the target cell. Using whole protein A-bearing staphylococci to detect IgG binding to peripheral blood and bone marrow slide preparations, Harmon and co-workers demonstrated a relationship between severity of neutropenia and the immaturity of marrow cells to which autoantibody would bind.²³ This assay as applied by others to cases of PWCA has been negative, however,^{16,17} perhaps because of an insufficient number of antigen-bearing target cells. Nevertheless, Levitt and others have provided strong evidence for IgG antibody mediation of idiopathic and drug-associated PWCA by demonstrating inhibition of CFU-GM growth in in vitro clonogenic assays of human marrow.¹⁶⁻¹⁹ Various studies have likewise suggested the presence of antibodies to RBC and platelet precursors in certain syndromes.^{11,12}

Because HL-60 cells appear to express the appropriate antigens, these readily available cells can be used to detect

antibodies to immature granulocyte precursors in a simple and quantitative manner. These cells can also be used as a source for specific identification of antigens by immunoprecipitation and/or immunoblotting procedures. This model may also be useful in analyzing disorders involving other cell types (eg, pure RBC aplasia, immune thrombocytopenia, aplastic anemia, vasculitides) by using other available human cell lines. Classification of syndromes of autoantibody-mediated granulocytopenia according to antigen targets will facilitate the analysis of pathophysiology and clinical characteristics. Antibodies to peripheral blood granulocytes with differing *in vitro* characteristics have been reported in association with many drugs and diseases, including rheumatoid arthritis, procainamide-related and idiopathic lupus, LGL, Hodgkin's disease, and other conditions.²²⁻³² The classification of antigranulocyte antibodies based on the binding ratio to mature v immature myeloid cells is another step toward identification of antigen specificities, which may have significant prognostic and therapeutic implications.

Studies using other human cell lines and correlation with serum effects on *in vitro* clonogenic assays of marrow progenitors may be useful in determining whether antigens not present on HL-60 cells (eg, HLA-DR) may be involved in some cases of antibody mediated myeloid hypoplasia and to what extent antibody binding to cell-line targets predicts inhibition in assays using bone marrow cells. These studies, though, could not be expected to detect antibody-mediated marrow damage due to very low-titer (predominantly marrow cell-bound) antibodies, or antibodies to antigens that are

sparse or only transiently expressed on target cells. Moreover, the demonstration of antibody binding to granulocyte precursors and/or serum inhibition in marrow progenitor assays can only suggest a possible mechanism of marrow injury and may not be sufficient to direct therapy.

In this report, we describe three patients with chronic idiopathic myeloid hypoplasia and the use of HL-60 cells to detect antibodies to immature myeloid cells in these and other patients in whom we did not detect antibodies to mature granulocytes. We also report the binding of antigranulocyte antibodies to immature myeloid cells in a subset of patients with detectable antibody binding to mature granulocytes. In the patients studied, antibodies to immature granulocytes were associated with myeloid hypoplasia. The detection of such antibodies in elderly patients with "idiopathic" or "benign" neutropenia and in other disorders in which autoimmune phenomena occur is consistent with an antibody-mediated mechanism of neutropenia. Further studies based on the use of continuously cultured human cell lines are expected to contribute to a better understanding of the pathophysiology of autoimmune phenomena.

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Spectrin Is Associated With Membrane-Bound Actin Filaments in Platelets and Is Hydrolyzed by the Ca^{2+} -Dependent Protease During Platelet Activation

By Joan E.B. Fox, Clifford C. Reynolds, Jon S. Morrow, and David R. Phillips

We recently showed that platelets contain submembranous actin filaments that are linked to glycoprotein (GP) Ib on the plasma membrane. In the present study, experiments were performed to determine whether spectrin was associated with these filaments. The membrane-bound filaments were isolated from Triton X-100 (Sigma, St Louis) lysates of unstimulated platelets by differential centrifugation. Platelet spectrin was detected immunologically by using antibodies against human brain and RBC spectrin. Immunoblots showed that platelet spectrin consisted of two polypeptides (mol wt 240,000 and 235,000) that were similar in apparent mol wt to those of the α and β chains of brain spectrin but differed slightly from those of RBC spectrin (mol wt 240,000 and 220,000). Immunoprecipitation experiments identified platelet spectrin as two minor polypeptides migrating on sodium dodecyl sulfate (SDS)-polyacrylamide gels between actin-binding protein (mol wt 250,000) and the platelet polypeptide P235 (mol wt 235,000). Immunoblots of fractions isolated from Triton X-100-lysed platelets revealed that the α and β chains of

platelet spectrin were associated almost entirely with the actin filaments that were linked to the plasma membrane. Little spectrin was recovered in the Triton X-100-soluble fraction or with the actin filaments that were not membrane bound. During activation of platelets with thrombin or ionophore A23187, the α and β chains of spectrin were hydrolyzed, generating a major degradation product of mol wt 160,000 and a minor one of mol wt 170,000. These two hydrolytic products were also generated in Triton X-100 lysates incubated in the presence of Ca^{2+} but were not produced when lysates were treated with leupeptin, ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), or N -ethylmaleimide, known inhibitors of the Ca^{2+} -dependent protease. These experiments show that spectrin is a previously unidentified component of the membrane-bound actin filament network and that hydrolysis of spectrin by the Ca^{2+} -dependent protease may regulate the interactions of the filaments during platelet activation.

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PLATELETS HAVE a characteristic discoid shape that is rapidly lost when physiological agonists activate the platelets. The factors responsible for maintaining the discoid shape or directing the activation-induced changes are not well understood. Microtubules have been thought to be critical in regulating platelet shape,^{1,2} but the finding that agonists induce a change in shape even when microtubules are stabilized with taxol³ suggests that additional factors are involved.

We recently showed that platelets contain a peripheral layer of actin filaments that is linked by actin-binding protein to glycoprotein (GP) Ib on the plasma membrane.^{4,5} This membrane-bound layer of actin filaments is distinct from the rest of the cytoskeleton and might function as a membrane skeleton, stabilizing the shape of the unstimulated platelet.⁴

The only other cell known to contain a membrane skeleton that regulates the shape of the cell is the RBC (for review, see refs 6 and 7). In this cell, the major component of the membrane skeleton is spectrin, which consists of elongated heterodimers comprising an α chain (mol wt 240,000) and a β chain (mol wt 220,000). The heterodimers are associated head to head, forming tetramers. The tail regions of the spectrin tetramers bind to short actin filaments, cross-linking these filaments into networks. Spectrin also links the filamentous network to the RBC plasma membrane through ankyrin, which connects spectrin to the membrane glycoprotein known as band 3. An additional linkage to the membrane may be provided by protein 4.1, which binds to spectrin and is also thought to be associated with the membrane glycoprotein glycophorin.⁸

Proteins immunologically similar to RBC spectrin,⁹⁻¹⁴ ankyrin,^{15,16} and protein 4.1¹⁷⁻²¹ have been described in many nonerythroid cells, including platelets.^{15,19,21} This raised the question of whether these proteins might be associated with the membrane-bound actin filaments in platelets. In the present report, we (a) identify platelet spectrin as two

polypeptides of mol wt 240,000 and 235,000, (b) show that spectrin is selectively associated with the membrane-bound actin filaments, and (c) demonstrate that spectrin is hydrolyzed by the Ca^{2+} -dependent protease during platelet activation, when the cell undergoes dramatic changes in shape.

MATERIALS AND METHODS

Preparation of platelet suspensions. Venous blood was drawn from healthy adult donors, and platelets were isolated from it by centrifugation as described previously.^{4,22} When the platelet pellet was removed from the tube after each centrifugation, care was taken not to include any RBCs that were sometimes found tightly packed below the platelets. The washed platelets were finally resuspended at about 1×10^9 platelets/mL in Tyrode's buffer containing 138 mmol/L of sodium chloride, 2.9 mmol/L of potassium chloride, 12 mmol/L of sodium bicarbonate, 0.36 mmol/L of sodium phosphate, 5.5 mmol/L of glucose, 1.8 mmol/L of calcium chloride, 0.4 mmol/L of magnesium chloride, and 10 ng of prostacyclin/mL, pH 7.4. When platelets were to be subsequently activated by platelet agonists, prostacyclin was omitted from the Tyrode's buffer. Microscopic examination showed that no contaminating RBCs were present.

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To obtain platelets in which the surface glycoproteins were ^3H -labeled, washed platelets were labeled by the sodium meta-periodate/sodium [^3H]borohydride method, as described previously.⁴ Platelet counts were made with a Coulter counter.

Incubations. For studies of intact platelets, platelet suspensions were stirred with 0.1 National Institutes of Health (NIH) U of thrombin/mL (a generous gift from Dr John W. Fenton II of the New York Department of Health, Albany) or with 1.0 $\mu\text{mol/L}$ of ionophore A23187 (Calbiochem, San Diego) as described previously.²³

For studies of lysed platelets, lysates were prepared by adding to platelet suspensions an equal volume of buffer containing 2% Triton X-100 (Sigma, St Louis), 100 mmol/L of Tris-HCl, 100 mmol/L of benzamide, and 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF) (Sigma), pH 7.4. When indicated, leupeptin (Vega Biotechnologies, Tucson, AZ), ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA), or N -ethylmaleimide (Sigma) was included in the buffer to inhibit activity of the Ca^{2+} -dependent protease. Lysates were incubated at 25 °C, and incubations were terminated by the addition of sodium dodecyl sulfate (SDS)-containing buffer.

Isolation of subcellular fractions. Platelets (500- μL portions) were lysed by addition of an equal volume of ice-cold buffer containing 2% Triton X-100, 10 mmol/L of EGTA, and 100 mmol/L of Tris-HCl, pH 7.4. In some experiments, as indicated in the text, actin filament depolymerization was induced by omitting EGTA from the lysis buffer or by omitting EGTA and including DNase I (2 mg/mL, Boehringer-Mannheim, Indianapolis). When EGTA was omitted, leupeptin (2 mg/mL) was included to inhibit activity of the Ca^{2+} -dependent protease. When DNase I was included, 2 mmol/L of PMSF and 100 mmol/L of benzamide were added to the lysis buffer to inhibit any protease activity present in the DNase I. Lysates were centrifuged at 100,000 g at 4 °C for 3 hours to obtain the Triton X-100-insoluble actin filaments and the Triton X-100-soluble fraction. To separate the membrane-bound filaments from the rest of the actin filaments, lysates were first centrifuged at 15,600 g at 4 °C for 4 minutes. The bulk of the actin filaments sedimented at these g forces. The membrane-bound filaments remained in the supernatant, however, and were subsequently isolated by centrifugation at 100,000 g at 4 °C for 3 hours.

SDS-polyacrylamide gel electrophoresis (PAGE). For analysis on SDS-polyacrylamide gels, 3 vol of sample were solubilized by addition of 1 vol of a buffer containing 8% SDS (wt/vol), 20% 2-mercaptoethanol (vol/vol), 40% glycerol (vol/vol), 0.008% bromophenol blue (wt/vol), 250 mmol/L of Tris-HCl, 20 mmol/L of EGTA, and 4 mg of leupeptin/mL, pH 6.8. Insoluble fractions were solubilized in the same buffer diluted four times with distilled water. All samples were incubated at 60 °C for 5 minutes to facilitate solubilization. Protein was electrophoresed through slab gels according to the method of Laemmli,²⁴ using 3% acrylamide in the stacking gel and either 5% or 7.5% acrylamide in the resolving gel. For detection of total protein, gels were stained with Coomassie brilliant blue or by a silver staining method.²⁵ For detection of ^3H -labeled glycoproteins by fluorography, gels were treated with EN ^{3} HANCE (New England Nuclear, Boston), dried, and exposed to x-ray film as described previously.⁴ Spectrin was detected on immunoblots as described below.

Antibody production and characterization. RBC ghosts were isolated and washed as described.²⁶ Spectrin was extracted from ghosts at 37 °C in a low-ionic-strength buffer and was further purified by gel filtration on Sepharose 4B as described by Tyler and colleagues.²⁷ Purified spectrin was electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide. The gel was stained briefly (~5 minutes) with Coomassie brilliant blue, and the two major bands of mol wt 240,000 and 220,000 (corresponding to the α

and β chains of spectrin) were cut out of the gel in a narrow strip. The gel strip was washed, homogenized, and injected into rabbits as described previously.²⁸ Antiserum was isolated by centrifugation of clotted blood for 20 minutes at 30,000 g. The immunoglobulin G (IgG) fraction was precipitated two times at 4 °C with 40% ammonium sulfate, dialyzed into a buffer containing 100 mmol/L of sodium chloride, 0.02% sodium azide, and 10 mmol/L of sodium phosphate, pH 7.4, and stored at 4 °C.

Antibodies against human brain spectrin were raised as described.²⁹ Monoclonal antibody against the β chain of RBC spectrin was kindly provided by Dr Stanley Schrier of Stanford University. Antibodies against actin-binding protein and glycocalicin (the water-soluble hydrolytic product of GP Ib) were raised as described previously.^{4,28}

Antibody specificities were characterized on immunoblots as described previously.^{28,30} The specificities of the affinity-purified actin-binding protein antibodies and the glycocalicin antibodies have been described.^{4,28} Antibody concentrations were determined by their absorbance at 280 nm using an $E_{1\text{cm}}^{1\%}$ value of 14.0.

Immunoprecipitation of proteins from platelet lysates. Platelet suspensions (1 to 2×10^9 platelets/mL) were lysed by the addition of an equal volume of ice-cold buffer containing 2% Triton X-100, 10 mmol/L of EGTA, 2 mg of leupeptin/mL, and 100 mmol/L of Tris-HCl, pH 7.4. In some experiments, DNase I (1 mg/mL) was included in the lysis buffer to depolymerize actin filaments and release filament-associated proteins into the Triton X-100-soluble fraction. When DNase I was present, PMSF (2 mmol/L) and benzamide (100 mmol/L) were included in the lysis buffer and the leupeptin concentration was increased to 4 mg/mL. Filamentous actin remaining in the Triton X-100 lysates was removed by centrifugation of the lysates at 100,000 g at 4 °C for 3 hours. Proteins were immunoprecipitated from 1-mL portions of the Triton X-100-soluble fraction as described previously.⁵ Immunoprecipitated protein was solubilized with 70 μL of an SDS-containing buffer and analyzed on SDS-polyacrylamide gels.

RESULTS

Characterization of spectrin antibodies. RBC ghosts and washed platelet suspensions were each solubilized in SDS-containing buffer and electrophoresed on SDS-polyacrylamide gels containing 7.5% acrylamide. The polypeptides were transferred electrophoretically to nitrocellulose paper, which was then incubated with antibodies against spectrin. Figure 1 shows that antibodies against both RBC spectrin and brain spectrin reacted with the α chain of mol wt 240,000 and the β chain of mol wt 220,000 of RBC spectrin (Fig 1A and B, lane 1). The antibodies also reacted with several minor polypeptides of lower mol wt present in the RBC ghosts, an observation made previously by others.⁹ Antibodies against RBC spectrin were raised in three different rabbits. Two of the antisera did not react with any platelet polypeptide on immunoblots. As shown in Fig 1, however, one antiserum reacted with a closely spaced doublet in platelets (Fig 1A, lane 2). The doublet had a mol wt of 240,000, as indicated by its comigration with the α chain of mol wt 240,000 of spectrin in RBC ghosts. A doublet of similar mol wt was also recognized in platelets by the antibodies against brain spectrin (Fig 1B, lane 2). There was no indication of a polypeptide in platelets that comigrated with the β chain of mol wt 220,000 of RBC spectrin. A monoclonal antibody against the β chain of RBC spectrin did not react with any platelet polypeptide (Fig 1C, lane 2).

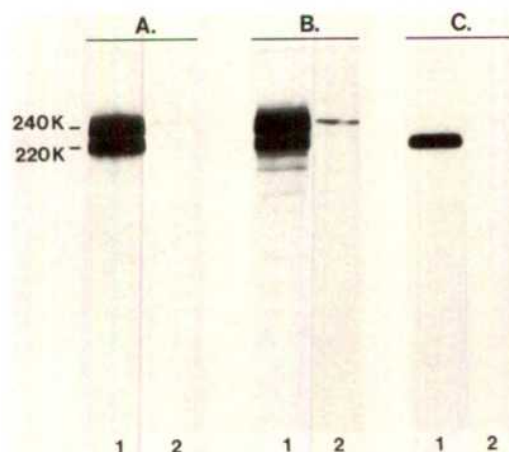


Fig 1. Characterization of spectrin antibodies. RBC ghosts ($\sim 25 \mu\text{g}$ of protein) (lane 1 in each panel) and washed platelet suspensions ($\sim 75 \mu\text{g}$ of protein) (lane 2 in each panel) were each solubilized in sodium dodecyl sulfate (SDS)-buffer and electrophoresed on SDS-polyacrylamide gels containing 7.5% acrylamide. The polypeptides were transferred to nitrocellulose paper, which was incubated with antibodies against (A) RBC spectrin (final concentration, $210 \mu\text{g}/\text{mL}$), (B) brain spectrin (final concentration, $120 \mu\text{g}/\text{mL}$), and (C) a monoclonal antibody against the β chain of RBC spectrin (final concentrations, $110 \mu\text{g}/\text{mL}$ for RBC polypeptides and $220 \mu\text{g}/\text{mL}$ for platelet polypeptides). Antigen-antibody complexes were detected by autoradiography after incubation of the nitrocellulose paper with ^{125}I -labeled Protein A.

To resolve the α and β chains of platelet spectrin, platelet polypeptides were electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide. Figure 2A shows that the α chain of platelet spectrin comigrated with the α chain of mol wt 240,000 of RBC spectrin, whereas the β chain migrated to a point between the α and β chains of RBC spectrin, with a mol wt of 235,000. These were the only two polypeptides that reacted specifically with the spectrin antibodies. Although a few additional minor bands were apparent (Fig 2A), these represented nonspecific binding of the antibody, as indicated by their presence in control blots as well (Fig 2B).

Immunoprecipitation of platelet spectrin. Antibodies against both RBC spectrin and brain spectrin caused immunoprecipitation of two polypeptides of spectrin from platelet lysates (Fig 3A, lanes 3 and 4). The two polypeptides were precipitated from platelet lysates even by batches of RBC spectrin antibodies that did not interact with platelet spectrin on immunoblots. The polypeptides were present in very small amounts and were barely detectable over the background of other proteins that were recovered nonspecifically in the immunoprecipitates. Their identification as platelet spectrin was indicated by their absence from immunoprecipitates obtained with normal rabbit IgG (Fig 3A, lane 2), however, and their identification was confirmed on immunoblots showing that the two immunoprecipitated proteins reacted with antibodies against brain spectrin (Fig 3B). The α chain of platelet spectrin comigrated with the α chain of mol wt 240,000 of RBC spectrin, whereas the β chain had a mol wt that was higher than that of both the β chain of RBC spectrin and the platelet polypeptide of mol wt 235,000 (P235) (Fig 3A, lanes 3 and 4 as compared with lanes 5 and 6).

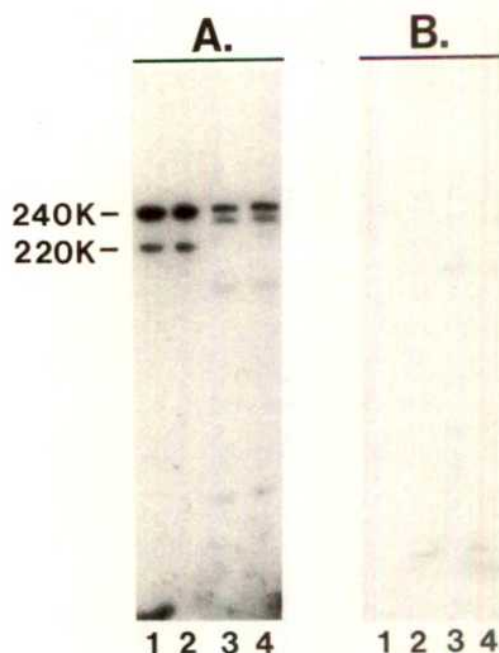


Fig 2. Detection of RBC and platelet spectrin on immunoblots of 5% acrylamide gels. RBC ghosts ($\sim 0.5 \mu\text{g}$ of protein) (lanes 1 and 2 in each panel) and washed platelet suspensions ($\sim 60 \mu\text{g}$ of protein) (lanes 3 and 4 in each panel) were solubilized in sodium dodecyl sulfate (SDS)-containing buffer. Solubilized proteins were electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide, transferred to nitrocellulose paper, and incubated with antibodies against (A) brain spectrin (final concentration, $120 \mu\text{g}/\text{mL}$) or (B) an equal concentration of normal rabbit serum. Antigen-antibody complexes were detected by autoradiography after incubation of the nitrocellulose paper with ^{125}I -labeled Protein A.

To identify which of the polypeptides in whole platelets represented spectrin, platelets were solubilized in SDS-buffer and then electrophoresed through SDS-polyacrylamide gels containing 5% acrylamide. Most one-dimensional gel systems resolve the two major high-mol-wt proteins actin-binding protein (mol wt 250,000) and P235 (mol wt 235,000), but not additional polypeptides in this mol-wt region. By using 5% acrylamide and running the gels until the dye front was well off the bottom of the gel, we were able to resolve two additional polypeptides that were visible on Coomassie brilliant blue-stained gels at points between actin-binding protein and P235 (Fig 3, lane 1). These polypeptides migrated to the same position on the gels as the two polypeptides precipitated by the spectrin antibodies. They were not present in platelet lysates from which spectrin had been immunoprecipitated (data not shown). We conclude that these two additional polypeptides represent platelet spectrin. The two polypeptides of platelet spectrin were present in approximately equal amounts and represented very minor components as compared with actin-binding protein and P235.

Association of spectrin with actin filaments in platelets. To determine whether spectrin was associated with actin filaments, platelets were lysed with Triton X-100 and

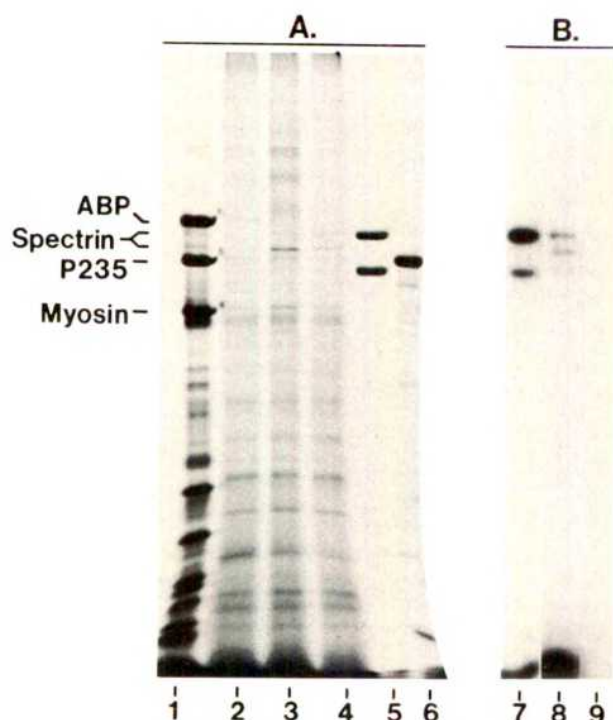


Fig 3. Immunoprecipitation of platelet spectrin. Platelets were solubilized directly into sodium dodecyl sulfate (SDS)-containing buffer (lane 1) or were lysed with an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, 100 mmol/L of Tris-HCl, 4 mg of leupeptin/mL, 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L of benzamide, pH 7.4. The Triton X-100-soluble fraction was obtained by centrifugation of the lysate at 100,000 g for 3 hours at 4 °C. The precipitate resulting from immunoprecipitation of 650 μ L of the Triton X-100-soluble fraction with 180 μ g of normal rabbit IgG/mL (lane 2), 180 μ g of RBC spectrin antibodies/mL (lane 3), or 150 μ g of brain spectrin antibodies/mL (lanes 4 and 8) was solubilized in SDS-containing buffer. Solubilized proteins were electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide. Lanes 5 and 7 contain purified RBC spectrin, and lane 6 contains platelet P235 purified as described previously.²⁸ Lane 9 contains the supernatant remaining after precipitation of platelet lysate with brain spectrin antibodies. In panel A, proteins were detected with Coomassie brilliant blue stain. In panel B, spectrin was detected on Western blots using antibodies against brain spectrin as described in the legend to Fig 1. ABP, actin-binding protein; P235, platelet polypeptide of mol wt 235,000 that appears to be similar to talin.³¹

the actin filaments were sedimented at g forces known to sediment all of the filamentous actin (100,000 g for 3 hours). Analysis of the Triton X-100-insoluble and -soluble fractions showed that spectrin was primarily recovered along with the Triton X-100-insoluble actin filaments (Fig 4A, lane 2 compared with lane 3). Because several other platelet proteins in addition to actin filaments are inherently insoluble in Triton X-100,⁴ the effect of depolymerization of the actin filaments on the Triton X-100 insolubility of spectrin was examined. Two depolymerizing agents were used: Ca^{2+} and DNase I. The addition of Ca^{2+} causes depolymerization of most of the actin filaments in platelet lysates, but not of the small pool of filaments that is membrane bound.⁴ Figure 4B shows that Ca^{2+} had little effect on the recovery of

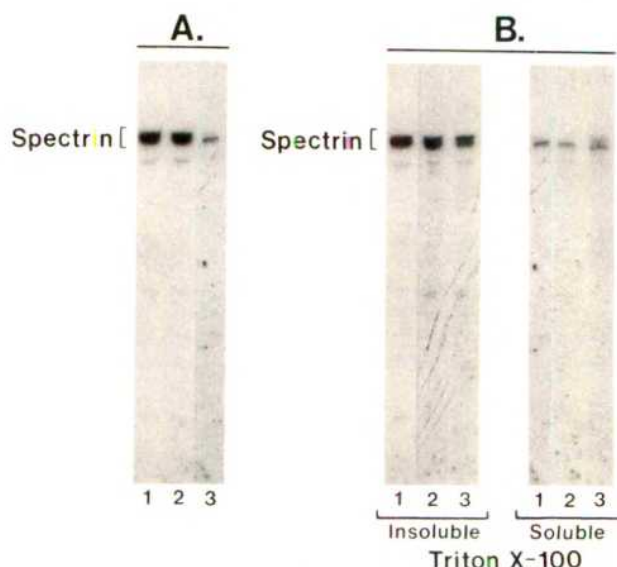


Fig 4. Recovery of spectrin with Triton X-100-insoluble actin filaments. (A) Platelets were solubilized directly into sodium dodecyl sulfate (SDS)-containing buffer (lane 1) or were lysed by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, and 100 mmol/L of Tris-HCl, pH 7.4. Lysates were centrifuged at 100,000 g at 4 °C for 3 hours. Total platelets (30 μ L) or fractions originating from 30 μ L of platelets were analyzed on SDS-polyacrylamide gels containing 5% acrylamide (Triton X-100-insoluble [lane 2] and -soluble [lane 3]). Spectrin was detected on immunoblots using antibodies against brain spectrin as described in the legend to Fig 1. (B) Platelet suspensions were lysed by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, and 10 mmol/L of Tris-HCl, pH 7.4 (lanes 1), the same buffer lacking a Ca^{2+} chelator but containing leupeptin 2 mg/mL to inhibit activity of the Ca^{2+} -dependent protease (lanes 2), and the same buffer lacking a Ca^{2+} chelator but containing leupeptin 2 mg/mL and DNase I (2 mg/mL), 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L of benzamide (lanes 3). Triton X-100-insoluble and -soluble fractions were isolated and analyzed for the presence of spectrin as described for panel A.

spectrin in the Triton X-100-insoluble fraction (lane 2 compared with lane 1). In contrast to Ca^{2+} , DNase I depolymerizes both cytoplasmic filaments and membrane-bound filaments.⁴ When DNase I was present at concentrations shown previously to depolymerize ~40% of the total actin filament content and release ~20% of GP Ib from the Triton X-100-insoluble fraction,⁴ the recovery of spectrin in the Triton X-100-insoluble fraction was also reduced (Fig 4B, lane 3 compared with lane 1). The differential effect of Ca^{2+} and DNase I on the recovery of spectrin in the Triton X-100-insoluble fraction suggests that spectrin is selectively associated with the membrane-bound actin filaments in platelets.

An additional characteristic of the membrane-bound filaments is that they can be separated from other platelet actin filaments by differential centrifugation of platelet lysates.⁴ Figure 5A (lane 2) shows that many of the actin filaments in platelets can be sedimented from Triton X-100 lysates by low-speed centrifugation. Little GP Ib is associated with these filaments (Fig 5B, lane 2). The membrane-bound actin filaments require higher speed centrifugation to be sedi-

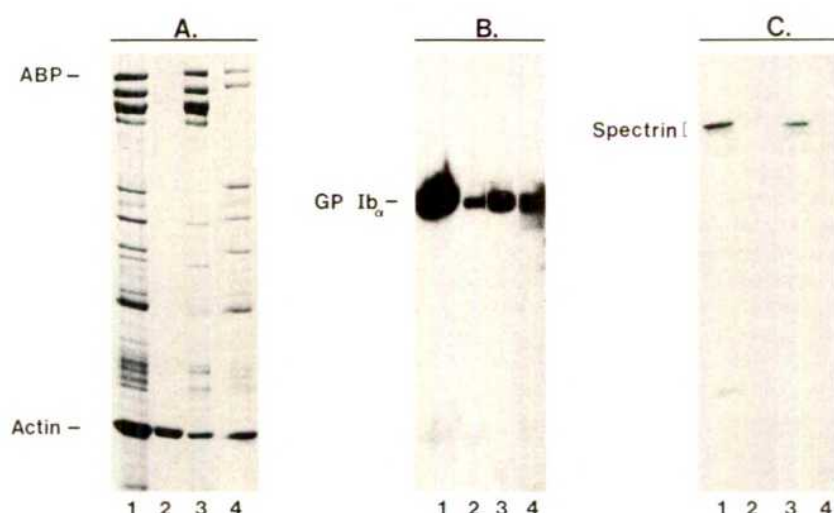


Fig 5. Recovery of spectrin with the membrane-bound actin filaments. Platelets, surface labeled by the sodium metaperiodate/sodium [^3H]borohydride method, were suspended in Tyrode's buffer and solubilized directly into sodium dodecyl sulfate (SDS)-containing buffer (lane 1 in each panel) or lysed by addition of an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, and 100 mmol/L of Tris-HCl, pH 7.4. Lysates were centrifuged at 15,600 g at 4 °C for 4 minutes, and the Triton X-100 supernatant was centrifuged at 100,000 g for a further 3 hours. The sediments obtained at low speed (lane 2 in each panel) and high speed (lane 3 in each panel) and the supernatant obtained at high speed (lane 4 in each panel) were analyzed on SDS-polyacrylamide gels containing 7.5% acrylamide (A) or 5% acrylamide (B and C). Polypeptides were detected with Coomassie brilliant blue stain (A), ^3H -labeled glycoproteins were detected by fluorography (B), and spectrin was detected on immunoblots using antibodies against brain spectrin (C). The protein analyzed in each lane originated from 30 μL of whole platelets. ABP, actin-binding protein; GP Ib, glycoprotein Ib.

mented (100,000 g for 3 hours). At this g force, there is increased sedimentation of several platelet proteins, including actin filaments and actin-binding protein (Fig 5A, lane 3) and GP Ib (Fig 5B, lane 3). This property of the membrane-associated actin filaments was used to provide further evidence that spectrin was selectively associated with these filaments. Figure 5C shows that, like GP Ib, little spectrin was associated with the actin filaments that could be sedimented from Triton X-100 lysates at low g forces (lane 2). Spectrin, like GP Ib, was primarily associated with the actin filaments requiring high-speed centrifugation to be sedimented (Fig 5C, lane 3).

It has been shown that actin-binding protein links actin filaments and GP Ib on the plasma membranes of platelets.⁵ To test whether spectrin was a component of this actin-binding protein-GP Ib linkage, actin filaments in platelet lysates were depolymerized with DNase I. The lysates were centrifuged at 100,000 g for 3 hours, and the resulting supernatant, which was free of actin filaments and contained proteins released from filaments during depolymerization, was used in immunoprecipitation experiments. Figure 6 shows that the actin-binding protein-GP Ib complex was precipitated from the high-speed supernatant by actin-binding protein antibodies (Fig 6A and 6B, lane 1) and by GP Ib antibodies (Fig 6A and 6B, lane 2). Spectrin antibodies precipitated spectrin (Fig 6A, lane 4) but not actin-binding protein (Fig 6A, lane 4) or GP Ib (Fig 6B, lane 4), indicating that spectrin is not a component of the actin-binding protein-GP Ib complex.

Hydrolysis of spectrin during platelet activation. Figure 7 shows that activation of platelets with the divalent cation ionophore A23187 resulted in hydrolysis of spectrin. The major degradation product was a polypeptide of mol wt

160,000. A minor degradation product of mol wt 170,000 was also apparent. The same degradation products were generated when platelets aggregated in response to thrombin (Fig 8). The major degradation product was apparent within seconds of addition of 0.1 NIH U of thrombin per milliliter. The amount of hydrolysis increased with time after thrombin was added (Fig 8, lanes 1 through 10). Hydrolysis was dependent on aggregation of platelets; it was not detected if platelets were prevented from aggregating by failure to stir the suspensions (data not shown).

Hydrolysis of spectrin by the Ca^{2+} -dependent protease in platelet lysates. It has been shown that the Ca^{2+} -dependent protease within platelets is activated when platelets aggregate after stimulation by thrombin, collagen, or ionophore A23187.²³ To determine whether hydrolysis of spectrin within aggregated platelets resulted from activity of the Ca^{2+} -dependent protease, platelets were lysed with Triton X-100 under conditions in which the Ca^{2+} -dependent protease was active. The degradation products produced in lysates were compared with those produced in intact cells. Figure 9A shows that spectrin was rapidly hydrolyzed. The major degradation product had a mol wt of 160,000. Products with a mol wt of 210,000 and 170,000 were also generated. These three new polypeptides arose from activity of the Ca^{2+} -dependent protease, as indicated by the inhibition of their generation by leupeptin and EGTA (Fig 9A, lanes 11 and 12). *N*-Ethylmaleimide, another inhibitor of the Ca^{2+} -dependent protease,³² also inhibited hydrolysis of spectrin (Fig 9A, lane 13). This inhibition, however, even at a concentration of 5 mmol/L of *N*-ethylmaleimide, was not as complete as with the other two inhibitors. Silver staining of SDS-gels showed that actin-binding protein and P235, the two major substrates of the platelet Ca^{2+} -dependent protease

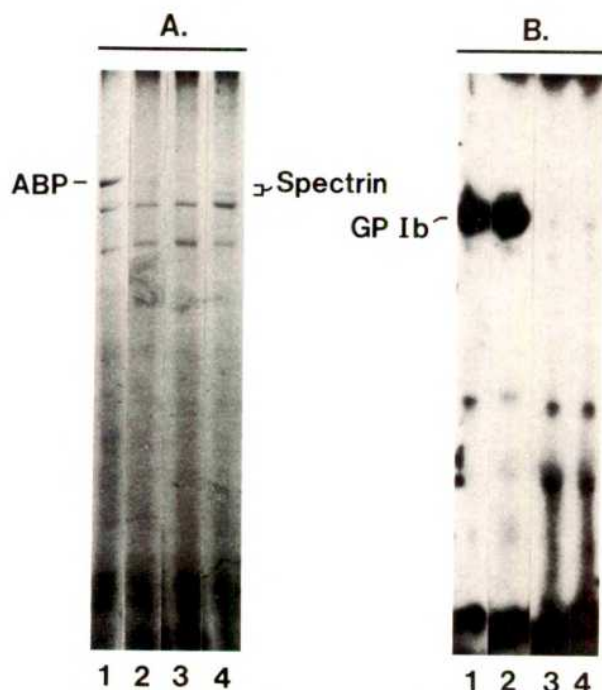


Fig 6. Demonstration that spectrin is not a component of the actin-binding protein-GP Ib complex. Platelet suspensions were lysed with an equal volume of a buffer containing 2% Triton X-100, 10 mmol/L of EGTA, 100 mmol/L of Tris-HCl, leupeptin 4 mg/mL, DNase I (1 mg/mL), 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L of benzamide, pH 7.4. Triton X-100-insoluble actin filaments were removed from the lysates by centrifugation at 100,000 g at 4 °C for 3 hours. Precipitates obtained from immunoprecipitation of 1 mL of the Triton X-100-soluble fraction with 4.5 μ g of actin-binding protein antibodies/mL (lane 1 in each panel), 1.3 mg of glycolaldehyde antibodies/mL (lane 2 in each panel), 1.3 mg of normal rabbit IgG/mL (lane 3 in each panel), and 1.3 mg of RBC spectrin antibodies/mL (lane 4 in each panel) were each solubilized in sodium dodecyl sulfate (SDS)-containing buffer. Solubilized proteins were electrophoresed on gels containing 5% acrylamide. Immunoprecipitated actin-binding protein and spectrin were detected with Coomassie brilliant blue stain (A); GP Ib was detected on immunoblots using antibodies against GP Ib (B). ABP, actin-binding protein; GP Ib, glycoprotein Ib.

identified previously,²⁸ were hydrolyzed at a rate similar to that of spectrin.

DISCUSSION

Proteins immunologically and functionally similar to RBC spectrin have been described in many nonerythroid tissues.⁹⁻¹⁴ The best characterized of these proteins, that found in brain, is referred to as fodrin, caldesmon, or brain spectrin. Brain spectrin, like RBC spectrin, is a tetramer in which $\alpha\beta$ heterodimers associate head to head and the tail regions bind to actin filaments.^{9,33} Despite the similarities in structure and function, the brain and RBC proteins do differ, as indicated by their peptide maps^{9,10} and the often weak reactivity of RBC spectrin antibodies against brain spectrin.¹⁰ The α chains, despite their similar molecular weights (~240,000), show different peptide maps; the β chains differ in both peptide maps and mol wt (~235,000 for brain spectrin as compared with ~220,000 for RBC spectrin). In

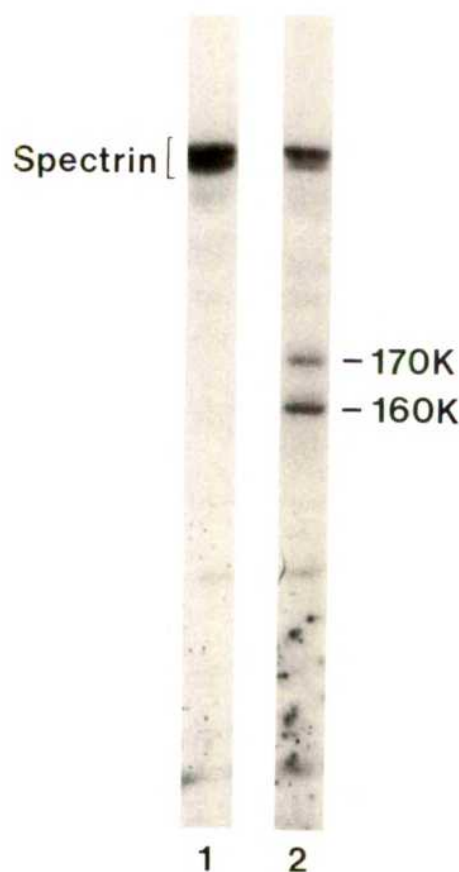
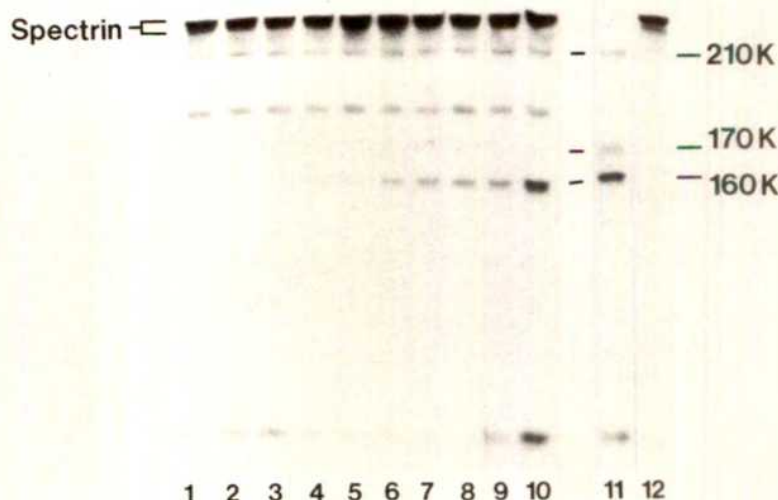


Fig 7. Hydrolysis of spectrin during activation of platelets with ionophore A23187. Platelet suspensions were solubilized in sodium dodecyl sulfate (SDS)-containing buffer (lane 1) or were stirred for 60 seconds at 37 °C with 1 μ mol/L of ionophore A23187 before solubilization (lane 2). Solubilized proteins were electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide, and spectrin was detected on immunoblots using antibodies against brain spectrin as described in the legend of Fig 1.

general, the spectrinlike proteins in mammalian nonerythroid cells appear to have mol wts similar to those of brain spectrin. It is becoming increasingly clear, however, that more than one isoform of nonerythroid spectrin can exist, even within the same tissue.^{34,35}

Early attempts, using antibodies against RBC spectrin, failed to detect spectrin in platelets.³⁶ More recent experiments detected a single polypeptide that comigrated on SDS-gels with the α chain of mol wt 240,000 of RBC spectrin.¹⁹ Because only one chain was detected in the latter study, it was not clear whether platelets contained the RBC form of spectrin or a form more closely related to brain spectrin. Because of the often low reactivity of RBC spectrin antibodies against spectrin in other tissues, we used brain spectrin antibodies to probe for spectrin in platelets. These antibodies detected two spectrin polypeptides both on SDS-gels of immunoprecipitated proteins and on immunoblots of total platelet proteins. The α chain of platelet spectrin comigrated with the α chain of mol wt 240,000 of RBC spectrin, whereas the β chain had a mol wt of 235,000. Platelets did not contain a polypeptide that comigrated with

Fig 8. Hydrolysis of spectrin during activation of platelets with thrombin. Platelet suspensions were solubilized in sodium dodecyl sulfate (SDS)-containing buffer (lane 1) or were stirred at 37 °C with thrombin 0.1 NIH U/mL for 5 seconds (lane 2), 10 seconds (lane 3), 15 seconds (lane 4), 20 seconds (lane 5), 30 seconds (lane 6), 45 seconds (lane 7), 60 seconds (lane 8), 5 minutes (lane 9), and 10 minutes (lane 10) before solubilization. Also shown are samples from another experiment in which unstimulated platelets were solubilized with Triton X-100 and lysates incubated in the presence of Ca^{2+} for 10 minutes (lane 11) or 0 minutes (lane 12) before solubilization. Solubilized proteins were electrophoresed on SDS-polyacrylamide gels containing 5% acrylamide, and spectrin was detected on immunoblots using antibodies against brain spectrin as described in the legend of Fig 1.



the mol wt 220,000 chain of RBC spectrin; neither did a monoclonal antibody against the β chain of RBC spectrin react with a polypeptide in platelets. We conclude, therefore, that platelets contain only one form of spectrin and that it is different from RBC spectrin. The mol wts of the α and β

chains of platelet spectrin are similar to those of brain spectrin.

In RBCs, spectrin is the primary component of the membrane skeleton, a structure beneath the plasma membrane that regulates the shape of the cell.^{6,7} The membrane skeleton

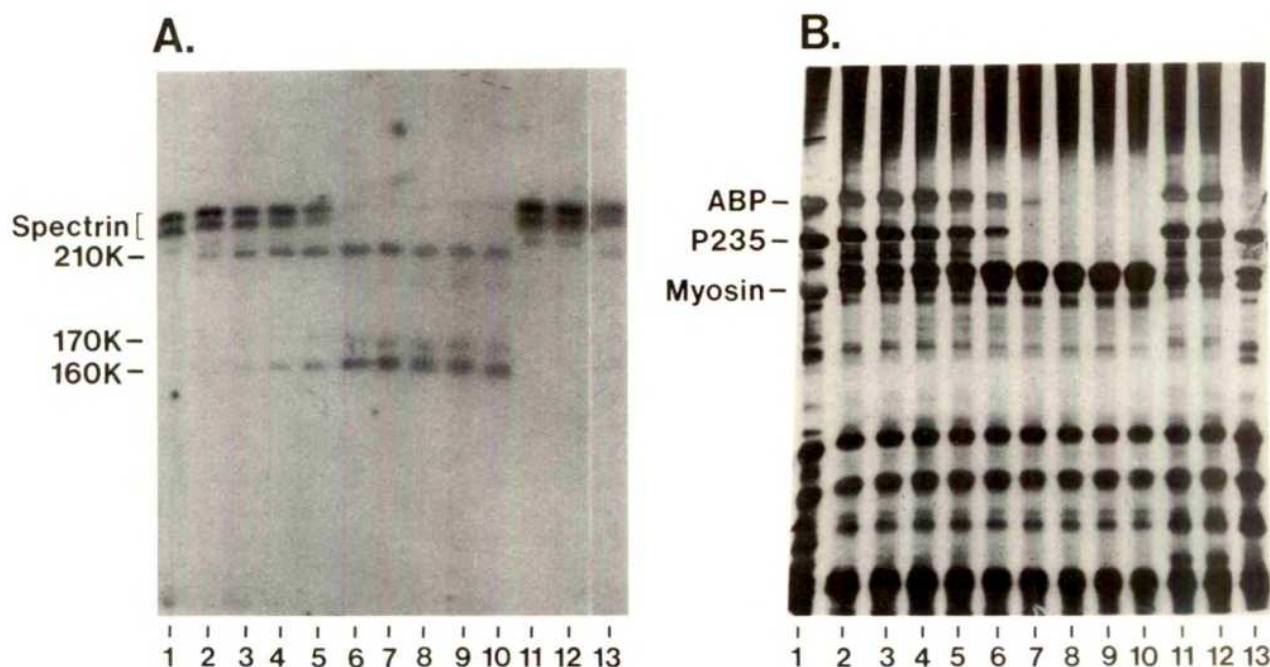


Fig 9. Hydrolysis of spectrin by the Ca^{2+} -dependent protease in platelet lysates. Platelet suspensions were lysed by addition of an equal volume of buffer containing 2% Triton X-100, 100 mmol/L of Tris-HCl, 2 mmol/L of phenylmethylsulfonyl fluoride (PMSF), and 100 mmol/L of benzamidinium, pH 7.4, and incubated at 25 °C. The incubation times for the lanes in both panels were as follows: 0 minutes (lane 1), 5 seconds (lane 2), 10 seconds (lane 3), 15 seconds (lane 4), 30 seconds (lane 5), 2 minutes (lane 6), 5 minutes (lane 7), 10 minutes (lane 8), 15 minutes (lane 9), and 30 minutes (lane 10). Incubations were also performed for 10 minutes in the presence of 5 mmol/L of EGTA (lane 11), leupeptin 1 mg/mL (lane 12), or 5 mmol/L of *N*-ethylmaleimide (lane 13). Incubations were terminated by addition of sodium dodecyl sulfate (SDS)-containing buffer, and polypeptides were separated by electrophoresis on SDS-polyacrylamide gels containing 5% acrylamide. Spectrin was detected on immunoblots using antibodies against brain spectrin (A); other polypeptides were detected by silver staining of the SDS-gels (B). ABP, actin-binding protein; P235, platelet polypeptide of mol wt 235,000 that appears to be similar to talin.³¹

is composed of short actin filaments that are cross-linked into networks by spectrin, which also links the networks to the plasma membrane. Immunofluorescence studies of several cell types,^{10,11,14} including platelets,¹⁹ have indicated that spectrin lies just under the plasma membrane in these cells too, raising the possibility that nonerythroid spectrin functions in the same way as RBC spectrin. We recently reported that a small pool of the filamentous actin in platelets is associated with glycoproteins on the plasma membrane.^{4,37} We suggested that the membrane-associated filaments may function like the membrane skeleton of RBCs in that they may play a role in regulating the shape of the platelet.⁴ Because the filaments that are attached to the membrane glycoproteins can be separated from other actin filaments within platelets,⁴ it was possible to test whether spectrin was associated with the membrane-bound filaments. The results reported here show that spectrin is indeed associated with the membrane-bound filaments in platelets.

A previous study⁵ has shown that one of the major components of the membrane-bound filamentous network in platelets is actin-binding protein. Like spectrin, it associates head to head, and the free tails of the polypeptide chains bind to actin filaments.³⁸ Like spectrin in RBCs, actin-binding protein in platelets connects the cross-linked filaments to glycoproteins on the plasma membrane.⁵ The present study shows that although spectrin is an additional component of the membrane-bound actin filament network in platelets, it is present in very small amounts as compared with actin-binding protein, which is one of the major platelet proteins. These observations suggest that the membrane-bound filamentous network in platelets differs from RBC membrane skeleton in that in platelets, actin-binding protein provides the major linkage between actin filaments and the plasma membrane, whereas in RBCs, spectrin is the major component of this linkage. If spectrin functions in platelets in the same way it does in RBCs, it would be expected to link actin filaments to a plasma membrane glycoprotein. Although spectrin does not appear to be involved in the linkage between actin-binding protein and GP Ib, it may provide an additional linkage between actin and an as-yet-unidentified component of the plasma membrane. Alternatively, spectrin in nonerythroid cells may function differently from its counterpart in RBCs. There is some evidence, for example, that spectrin may associate with microtubules.³⁹ The potential role of platelet spectrin in linking the membrane-associated filaments to the plasma membrane glycoproteins, microtubules, or other actin filaments awaits further study.

One of the initial responses of platelets to stimulation is their transformation from discs into irregular spheres.⁴⁰ Later, platelets undergo dramatic changes in shape as they spread on surfaces⁴¹ or extend long filopodia during the process of clot retraction.⁴² By analogy to the RBC, in which alterations in the membrane skeleton produce changes in cell shape,⁴³ the early changes in shape that platelets undergo in

response to activation could result from reversible modifications of components of the membrane-bound filamentous network. The later changes in the shape of the platelet membranes are so dramatic that the membrane-bound network would probably have to be disrupted for these changes to occur. Such a disruption has been shown to result from activation of the Ca^{2+} -dependent protease within platelets. This protease hydrolyzes actin-binding protein,²⁸ resulting in release of the actin filaments from their attachment to GP Ib on the plasma membranes.⁵ The results presented here show that platelet spectrin is also a substrate for the Ca^{2+} -dependent protease and is hydrolyzed during platelet activation with a time course similar to that reported for hydrolysis of actin-binding protein and P235.^{23,28} We reported that the Ca^{2+} -dependent protease is only activated in stimulated platelets if the platelets are allowed to aggregate.²³ A similar aggregation dependency for the hydrolysis of spectrin within platelets was detected in this study. In platelet lysates, hydrolysis of spectrin by the Ca^{2+} -dependent protease resulted in generation of three degradation products (mol wt 210,000, 170,000, and 160,000). In intact platelets, in which hydrolysis was more limited, the mol wt 160,000 product was the most readily detected. The mol wt of this major degradation product is similar to the mol wt 150,000 reported by Siman and co-workers for the product of hydrolysis of brain spectrin by purified Ca^{2+} -dependent protease.⁴⁴

Many of the proteins that are considered candidates for linkage proteins between actin filaments and plasma membranes in non-muscle cells (for example, actin-binding protein, vinculin, α -actinin, protein 4.1, and P235) are substrates for the Ca^{2+} -dependent protease.^{28,31,45-47} Furthermore, it was recently shown that the Ca^{2+} -dependent protease, previously thought to be a cytosolic enzyme,³² can bind to RBC membrane in an interaction that activates the enzyme.⁴⁶ These observations, together with the finding that activation of the Ca^{2+} -dependent protease during platelet activation results in hydrolysis of actin-binding protein and disruption of the interaction between actin filaments and GP Ib, emphasize the importance of the Ca^{2+} -dependent protease in regulating the interaction of actin filaments with plasma membranes. The finding that spectrin is an additional component of the membrane-bound filamentous network in platelets and that it is hydrolyzed by the Ca^{2+} -dependent protease during platelet activation suggests still another mechanism for regulation of the membrane-bound actin filaments during platelet activation.

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Leukemic Blast Cell Colony Formation in Semisolid Culture With Erythropoietin: A Case Report of Acute Poorly Differentiated Erythroid Leukemia

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The bone marrow of a patient with acute undifferentiated leukemia developed unique colonies after a 14-day culture in erythropoietin (EPO)-containing methylcellulose. The colonies consisted of 20 to 200 nonhemoglobinized large blast cells. Cytogenetic analysis of single colonies revealed hypotetraploid karyotypes with several marker chromosomes that were identical to those found in directly sampled bone marrow. The concurrently formed erythroid bursts showed only normal karyotypes. No leukemic colony formation was observed in other culture systems with either colony-stimulating activity (CSA) or phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM). The leukemic colonies exhibited a complete EPO-dose dependency similar to that of the patient's normal BFU-E. Although cytochemical and immunologic marker

studies of the bone marrow cells failed to clarify the cell lineage of the leukemic cells with extraordinarily large cell size, ultrastructural study revealed erythroid differentiation such as siderosome formation in the cytoplasm and ferritin particles in the rhopheocytosis invaginations. These findings indicate that the patient had poorly differentiated erythroid leukemia and that some of the clonogenic cells might respond to EPO in vitro. Corresponding to this biological feature, the leukemic cells were markedly decreased in number in response to repeated RBC transfusions, and partial remission was obtained. These observations suggest that erythroid leukemia distinct from erythroleukemia (M6) with a myeloblastic component, can develop as a minor entity of human acute leukemia.

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L EUKEMIC CELLS from patients with acute myeloid leukemia (AML) can form colonies composed of leukemic blasts in semisolid medium containing colony-stimulating factor (CSF)^{1,2} or phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM).³⁻⁶ Izaguirre and colleagues also developed a culture method containing PHA-stimulated T cell-conditioned medium (TCM) and obtained leukemic colonies from patients with acute lymphocytic leukemia (ALL).⁷ Recently, Dicke and co-workers reported a double-agar layer method with PHA and CSF in the underlayer and obtained leukemic colonies from patients with either AML or ALL.⁸ The clonogenic cells in these leukemic colonies were designated leukemic colony-forming units (L-CFU).^{9,10} Among various biological features of L-CFU so far elucidated, especially in the PHA-LCM system, self-renewal function as measured by secondary plating efficiency (PE2) appears to be of special importance because it defines the stem cell nature of at least a part of these cells.^{11,12} In this article, we describe the first instance of leukemic blast cell-colony formation in erythropoietin (EPO)-containing methylcellulose culture from a patient with acute undifferentiated leukemia with ultrastructural evidence of erythroid differentiation and distinct chromosomal aberration.

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MATERIALS AND METHODS

Case Report

The patient, a 50-year-old man, was admitted to a local hospital on September 10, 1983, because of a heel bone fracture. No abnormality was found in his peripheral blood, but pancytopenia developed rapidly during the next 2 months. He was transferred to our department on November 21, 1983. On admission, he was anemic and appeared acutely ill. No lymphadenopathy or splenomegaly was found. The liver was palpated 3 cm below the right costal margin. The hemoglobin (Hb) level was 5.8 g/dL. The WBC count was $3.6 \times 10^9/L$ with 52% mature neutrophils, 40% lymphocytes, 2% eosinophils, and 6% monocytes. The platelet count was $41 \times 10^9/L$. Initial bone marrow aspirate was highly cellular, with 31.6% abnormal blast cells, 1.0% myeloblasts, 5.9% promyelocytes, 6.8% myelocytes, 7.4% metamyelocytes, 32.8% mature neutrophils, 1.6% pronormoblasts, and 2.8% normoblasts. Megakaryocytes were decreased in number. The abnormal cells were extraordinarily large (30 to 40 μm in diameter), had a finely reticulated chromatin pattern, and moderate to strongly basophilic cytoplasm with some vacuoles but no granules (Fig 1). A few cells resembled pronormoblasts on May-Grunwald Giemsa (MG)-stained film. The patient was given 3 U of packed RBC for the next 3 days and recovered. After this transfusion, peripheral blood findings improved and were stable for ~40 days with Hb 8.3 g/dL and WBC $4.55 \times 10^9/L$; bone marrow showed a marked reduction of abnormal cell percentage to 3.4%, and erythroblastic elements were elevated to 65.6% (December 20, 1983). On January 6, 1984, however, leukemic cells in the bone marrow again increased to 30%. A low-dose cytosine arabinoside (ara-C) regimen (10 mg/day, continuous intravenous (IV) infusion for 16 days) was begun. A partial remission, with <5% abnormal cells, was obtained (February 8, 1984), but the leukemic cells began to increase again (February 20, 1984). The patient was further treated with the same regimen for 32 days. A second partial remission was obtained (April 10, 1984) but was transient, with a resultant increase of leukemic cells to 10% (April 24, 1984). A more intensive chemotherapy with ara-C and daunorubicin did not induce remission, and the leukemic cell proliferation became extensive, with rapidly increasing hepatosplenomegaly. The patient soon died of pneumonia and intestinal bleeding.

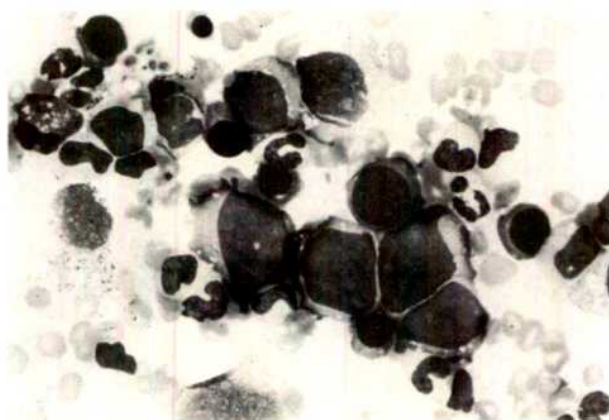


Fig 1. May-Grunwald Giemsa (MG) stain of the initial bone marrow (November 28, 1983) shows an increase (31.6%) of primitive blast cells with uniformly large cell sizes, a finely reticulated chromatin pattern, and several vacuoles. A few of them resemble normal pronormoblasts, exhibiting concentric nucleus and deeply basophilic cytoplasm with perinuclear halo (original magnification $\times 400$; current magnification $\times 264$). These abnormal blasts were negative for myeloperoxidase, specific and non-specific esterases, and hemoglobin stains. The bone marrow has well-preserved normal granulocytic elements, but those of the erythroid series are markedly reduced in number.

Cytological Analysis

Cytochemical stains used were myeloperoxidase (MPO),¹³ dual-esterase stain for naphthol-ASD chloroacetate esterase and α -naphthyl butyrate esterase,¹⁴ acid phosphatase,¹⁵ β -glucuronidase,¹⁶ periodic acid-Schiff (PAS),¹⁷ and Hb.¹⁸ Ultrastructural study of bone marrow cells was performed by routine double staining with uranyl acetate and lead citrate and cytochemical staining for MPO.¹⁹

For immunological marker analysis, several monoclonal antibodies such as J5 (common ALL antigen, CALLA, Coulter Immunology, Hialeah, FL); Leu 1 and HLA-DR (Becton Dickinson, Mountain View, CA), and OKM 1 (Ortho Pharmaceuticals, Raritan, NJ) were used. Terminal deoxynucleotidyl transferase (TdT) was detected by indirect immunofluorescence using anti-TdT antibody (Bethesda Research Labs, Gaithersburg, MD).

Colony Formation

CFU-E and BFU-E Assay.²⁰ Bone marrow buffy coat cells were collected after centrifugation at 1,200 rpm for 10 minutes. Cells (2×10^5) were plated in 1.0 mL of α -medium containing a final concentration of 0.88% methylcellulose, 30% fetal calf serum (FCS), 1% bovine serum albumin (BSA) and 1 U/mL of EPO (step III, Connaught, Canada) and cultured in a 35-mm Falcon plastic dish. For testing EPO-dose response by L-CFU, BFU-E, and CFU-E, EPO was added to final concentrations of 0, 0.1, 0.25, 0.5, and 1.0 U/mL, respectively. CFU-E and BFU-E were scored at day 7 and day 14, respectively.

CFU-GM Assay.²¹ Bone marrow light-density mononuclear cells (10^5) were cultured in a 35-mm Falcon plastic dish containing 1 mL of α -medium with 0.88% methylcellulose, 20% FCS, 10% giant cell tumor-conditioned medium (GTC-CM) (GIBCO, Grand Island, NY). Cell aggregates consisting of >20 cells were scored as colonies on day 8.

L-CFU assay.⁴ T cell-depleted bone marrow mononuclear cells (2×10^5) were plated in a 35-mm Falcon plastic dish containing 0.88% methylcellulose and 10% PHA-LCM. After 7-day incubation,

cell aggregates consisting of >20 cells were scored as colonies. This method allowed the detection of L-CFU colonies in 32 (82.5%) of 40 AML cases.

Cytogenetic Analysis

Bone marrow cells were processed directly for chromosomal preparation, and cytogenetic analysis was performed using standard methods and/or the G-banding method.²²

Chromosome analyses of individual colonies were performed by our own method described elsewhere.²³ In brief, individual colonies were aspirated and transferred into a droplet (10 μ L) of prewarmed 0.075 mol/L of KCl solution placed on a poly-L-Lysine-coated slide (Sigma Chemical, St Louis). After thorough mixing, the slide remained for 35 minutes at 37°C to allow the cells to attach. The slide was fixed, first with 30% fixative (3:1 methanol:acetic acid diluted with 0.075 mol/L of KCl), second with 20% ethanol diluted with 0.075 mol/L of KCl, and third with 100% fixative. The slide was then flame-dried and stained with Giemsa and/or Q-banded. This method yielded analyzable metaphases even from small colonies containing <40 cells. All laboratory examinations and in vitro assays were performed with informed consent from the patient.

RESULTS

Cytological Features of the Leukemic Cells

The undifferentiated, extraordinarily large leukemic cells were MPO⁻, both types of esterase⁻, acid phosphatase⁺ (86%), β -glucuronidase⁻, PAS⁻, and Hb⁻. They were also ultrastructurally MPO⁻. Immunological marker study disclosed CALLA⁻, Leu 1⁻, TdT⁻, OKM1⁻, and HLA-DR⁺ (12%). Transmission electron microscopic examination revealed distinct siderosome formation in the cytoplasm of $<5\%$ of the leukemic cells and fairly abundant rhopheocytotic invaginations and vesicles containing ferritin particles in half of the leukemic cells (Fig 2A, B, C).

Cytogenetic Findings in Bone Marrow Cells

Results of the serial cytogenetic studies are shown in Table 1. Abnormal hypotetraploid karyotypes were found on initial examination. Most of them had several identical marker chromosomes (Fig 2A). Repeated examinations revealed a considerable karyotypic instability (data not shown). Normal karyotypes were also present, but as a minor population. During the first partial remission, a clone with hypodiploid karyotypes was also observed. The relationship of the hypodiploid clone to the hypotetraploid clone could not be clearly explained. The hypotetraploid clone may represent the giant leukemic blast cells because they are known to have an excess amount of chromosomal materials.

Leukemic Blast Cell Colony Formation in EPO-Containing Culture

Erythroid colony assay of the bone marrow performed at the time of partial remission (January 6, 1984) disclosed bizarre colonies (Fig 3) consisting of 20 to 200 large non-hemoglobinized cells as well as well-hemoglobinized erythroid burst formation (Table 2). These two types of colonies reached maximum number on day 14 and did not form a single mixed colony. The cells from the bizarre colonies were uniformly large blast cells on MG-stained cytospin-smears

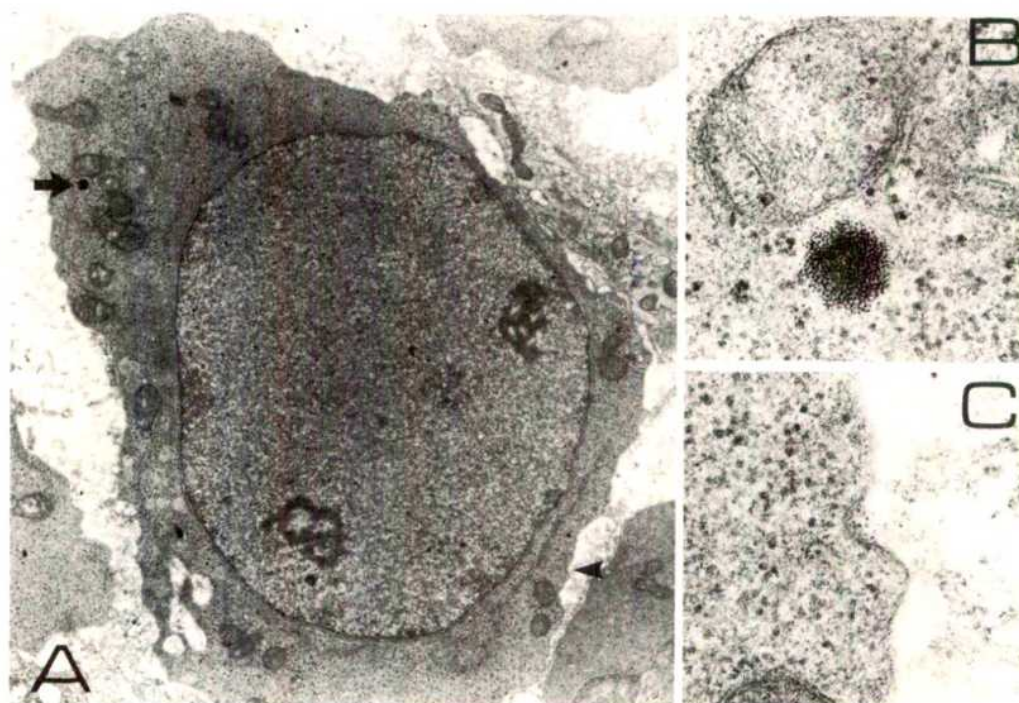


Fig 2. (A) Transmission electron microscopic features of a leukemic blast. The blast is large ($40\ \mu\text{m}$), has prominent nucleoli, fine chromatin structure without condensation, elongated endoplasmic reticulum, and occasional lysosomal granules and lipids (original magnification $\times 9,200$; current magnification $\times 5,796$). It possesses both siderosome ($\rightarrow$) and rhopheocytotic invagination ($\blacktriangleright$). (B) Higher magnification of the siderosome (original magnification $\times 76,000$; current magnification $\times 47,880$). (C) Higher magnification of the rhopheocytotic invagination with ferritin particles (original magnification $\times 76,000$; current magnification $\times 47,880$).

and resembled the leukemic cells in the bone marrow. They were negative for Hb, dual-esterase, and MPO stains. Cytogenetic analysis from individual day 14 colonies including normal-appearing erythroid bursts obtained from the relapsing bone marrow (February 20, 1984) was performed (Table 3). Karyotypes from the bizarre colonies revealed hypotetraploidy with several identical marker chromosomes, indicating that they belonged to a single abnormal clone (Fig 4B). Furthermore, these karyotypes carried marker chromosomes identical to those observed in the hypotetraploid karyotypes of the directly sampled bone marrow (Fig 4A). In contrast, karyotypes from erythroid bursts were all normal, showing 46, XY.

No leukemic colony formation was observed after cultur-

ing with GCT-CM or PHA-LCM. The data of serial colony assays are summarized in Table 2. The numbers of CFU-GM and erythroid bursts had recovered considerably at the time of partial remissions.

EPO-Dose Response by L-CFU

Figure 5 shows the EPO-dose-response curves for leukemic colonies and erythroid colonies from the bone marrow

Table 1. Serial Cytogenetic Study on Directly Sampled Bone Marrow Cells

Date	No. of Metaphases* Analyzed	Karyotypes		
		46, XY (%)	Hypodiploid (%)†	Hypotetraploid (%)‡
Nov 28, 1983	32	6 (19)	0 (0)	26 (81)
Dec 20, 1983	29	9 (31)	16 (55)	4 (14)
Jan 6, 1984	34	6 (18)	0 (0)	28 (82)
Feb 20, 1984	13	2 (15)	0 (0)	11 (85)
April 10, 1984	30	12 (40)	1 (3)	17 (57)
April 25, 1984	11	1 (9)	0 (0)	10 (91)

*All metaphases were photographed and karyotyped.

†Chromosome number ranging from 43 to 46; modality 45.

‡Chromosome number ranging from 66 to 89; modalities 77, 78, and 80 at different dates of cytogenetic examinations.

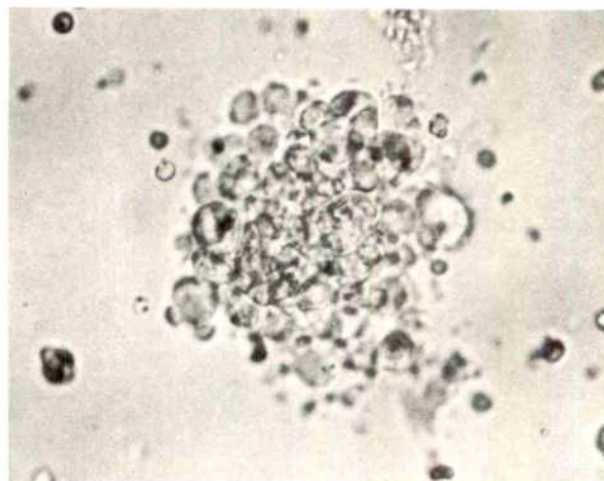


Fig 3. Leukemic blast cell colony observed in day 14 erythropoietin containing culture shows an aggregation of large nonhemoglobinized cells (original magnification $\times 200$).

Table 2. Serial Assays of Colony-Forming Cells

Cells	Nov 28, 1983	Jan 6, 1984	Feb 20, 1984	April 10, 1984	April 24, 1984
CFU-GM	90 ± 6	15 ± 2	1 ± 1	448 ± 14	56 ± 8
CFU-E	—	120 ± 20	—	121 ± 37	59 ± 18
BFU-E	—	54 ± 10	16 ± 1	80 ± 12	36 ± 8
L-CFU in EPO culture*	—	10 ± 3	15 ± 3	0	31 ± 2
L-CFU in PHA-LCM culture†	—	—	0	—	—

EPO, erythropoietin; PHA-LCM, phytohemagglutinin-stimulated leukocyte-conditioned medium. L-CFU, leukemic colony-forming unit. Each colony number shows the mean ± SD of triplicate cultures.

Normal value for each colony-forming unit; CFU-GM 117 to 210 (mean 160) per 1×10^5 light-density bone marrow cells; CFU-E 145 to 230 (mean 182) per 2×10^5 bone marrow buffy coat cells; BFU-E 72 to 122 (mean 94) per 2×10^5 bone marrow buffy coat cells.

*Counted on day 14 of culture.

†Counted on day 7 of culture.

specimen obtained on April 24, 1984. The response curve of L-CFU was similar to those of CFU-E and BFU-E but appeared to be slightly higher at EPO concentration of 0.1 U/mL. A few leukemic colonies with <30 cells developed spontaneously without EPO.

DISCUSSION

The extraordinarily large leukemic blast cells of this patient lacked evidence of differentiation toward specific cell lineage on conventional cytochemical and cell surface marker analyses. Ultrastructural investigation demonstrated, however, at least in some of the leukemic cells, distinct siderosome formation in the cytoplasm and ferritin particles in the rhopheocytotic invaginations on the cell surface membrane. These findings provide evidence of erythroid differentiation^{24,25} proceeding in the leukemic cell population. Recently, Reiffers and co-workers reported two similar cases of acute undifferentiated leukemia.²⁶ Clinical and hematologic features common to these three cases are (a) relatively advanced age (>50 years), (b) poor response to conventional chemotherapy, (c) low WBC count with minimal or absent leukemic blasts in the peripheral blood, (d) extraordinarily large leukemic cells, (e) high ploidy and instability of karyotype, (f) ultrastructural evidence of erythroid differentiation and (g) lack of simultaneous proliferation of peroxidase-positive myeloid precursor cells. These characteristics suggest the possibility that this type of acute leukemia is a clinical entity distinct from erythroleukemia (M6) with a myeloblastic component,²⁷ and may be a poorly differentiated type of acute erythremia.²⁸⁻³¹

It is not yet clear whether the immunologic markers of erythroid differentiation such as glycophorin A and spectrin can be demonstrated on these poorly differentiated leukemic cells. Detection of such markers using monoclonal or heterologous antibodies may reveal an erythroid nature of undiffer-

entiated leukemia.³²⁻³⁴ These markers were also demonstrated, however, on AML blasts³⁵ and K562 cells with simultaneous expression of myeloid antigen.³⁶ Therefore, combined ultrastructural and immunological analyses of future cases of undifferentiated leukemia must be conducted to clarify the kind of markers that provide the earliest and consistent evidence of erythroid differentiation in leukemic cells.

The bizarre nonhemoglobinized colonies that developed concurrently with the patient's normal erythroid bursts in EPO-containing culture apparently derived from the leukemic clone, as evidenced by the presence in single colonies of a common karyotypic abnormality identical to that of the leukemic clone in the bone marrow. The L-CFUs from our patient did not develop colonies in other culture systems with GCT-CM or PHA-LCM, indicating that the L-CFUs responded to EPO itself in the culture medium. There remains a minor possibility of response to another activity, however, such as burst-promoting activity (BPA) contaminating the partially purified EPO material. We found no report of leukemic blast cell colony formation in BFU-E assay or leukemic colonies expressing erythroid markers in other culture systems. Clonal culture studies in acute erythroleukemia (M6) are also few.^{37,38} Suda and co-workers examined colony formation in the bone marrow cells from four patients with erythroleukemia, using a plasma clot system containing EPO and Minden's PHA-LCM system.³⁷ They observed defective erythroid colony formation in all cases. They did not note blast cell colony formation in EPO-containing system, although the PHA-LCM system yielded blast cell colonies that evidenced granulocytic differentiation. Thus, their findings in erythroleukemia are distinct from our present observation.

The L-CFUs from our patient showed an almost complete dependency when EPO was added to the culture. The

Table 3. Cytogenetic Analysis from Single Colonies Growing in Erythroid Culture*

Colony	No. of Colonies Aspirated	No. of Colonies with an Analyzable Metaphase†	No. (%) of Colonies With a Karyotype of		
			46, XY	Hypodiploidy	Hypotetraploidy
BFU-E‡	24	11	11 (100%)	0	0
L-CFU‡	8	6	0	0	6 (100%)

*Culture for the bone marrow cells of February 20, 1984, when the leukemia began to relapse from partial remission.

†All metaphases were photographed and karyotyped.

‡Aspiration of each colony was performed on day 14 of culture. L-CFU: leukemic colony-forming unit.

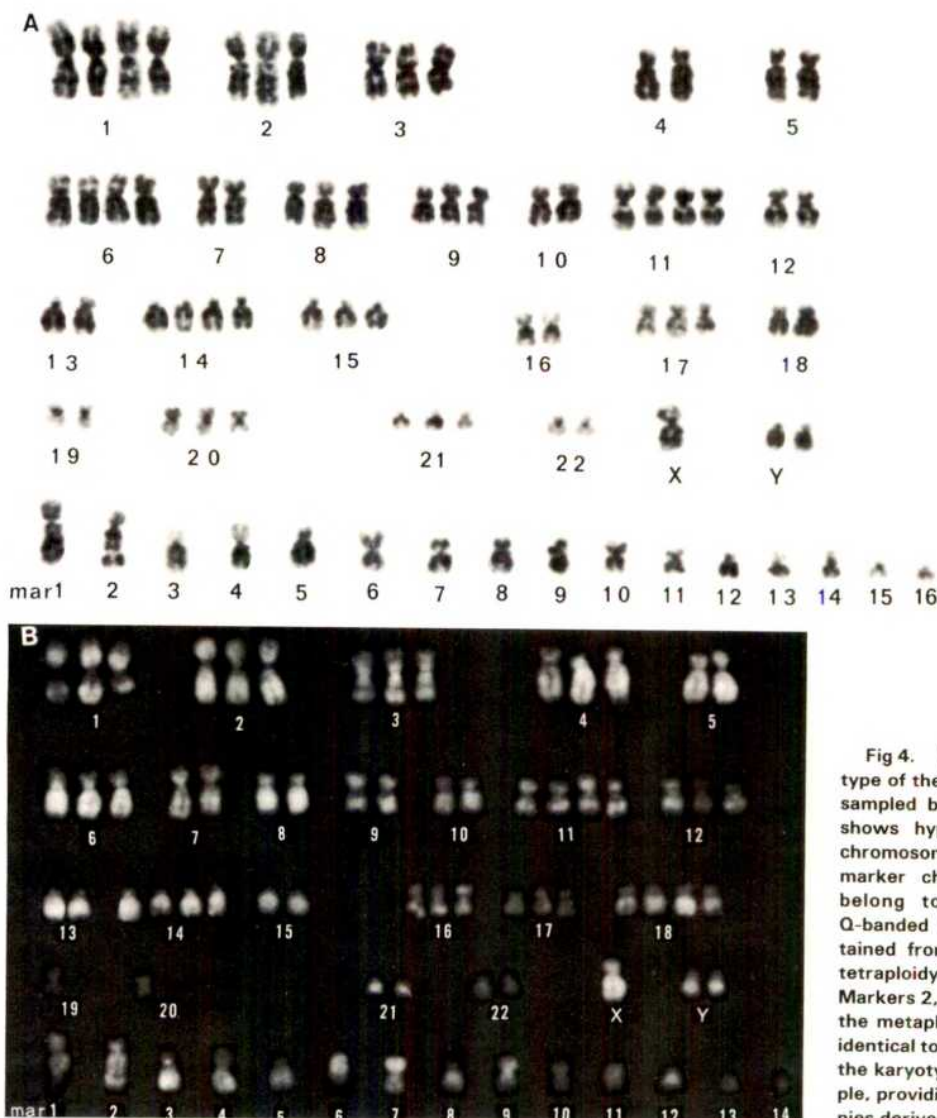


Fig 4. (A) Representative G-banded karyotype of the metaphases obtained from directly sampled bone marrow (November 28, 1983) shows hypotetraploidy and several marker chromosomes. Most of the metaphases carry marker chromosomes, indicating that they belong to a single abnormal clone. (B) Q-banded karyotype of the metaphase obtained from a leukemic colony shows hypotetraploidy and several marker chromosomes. Markers 2, 3, 4, 7, and 9 are common between the metaphases, and markers 2, 4, and 9 are identical to markers 2, 4, and 6, respectively, of the karyotype of the direct bone marrow sample, providing evidence that the blast cell colonies derive from the leukemic clone.

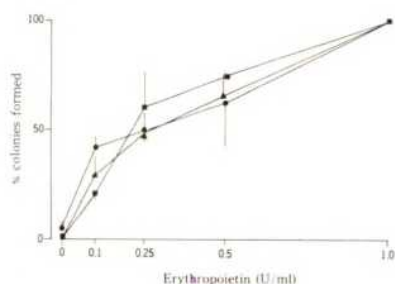


Fig 5. Erythropoietin (EPO) dose-response curves. The response curve of leukemic colony forming unit (●) is similar to those of normal BFU-Es (■) and CFU-Es (▲), but the L-CFUs show a slightly higher sensitivity to EPO at a low concentration of 0.1 U/mL. A few small leukemic colonies developed without EPO. The colony numbers at EPO 1.0 U/mL were regarded as 100%.

dose-response curve was similar to those of the patient's own normal BFU-Es and CFU-Es, but the L-CFUs showed a slightly higher sensitivity at the lowest dose of EPO. Theoretically, this hypersensitivity may explain the *in vivo* growth advantage of the leukemic clone over the normal erythroid progenitors. The reduction of the leukemic cell number in the bone marrow was rapid and marked after repeated transfusions, suggesting that a kind of feedback phenomenon occurred and induced a clinical partial remission without the use of chemotherapeutic agents. This observation indirectly supports the view that the leukemic cell proliferation *in vivo* was at least in part dependent on a given concentration of EPO. The feedback phenomenon in our patient mimics the marked erythroid depression inducible in patients with erythroleukemia by hypertransfusion.³⁹⁻⁴¹

Leukemic blast cell colony formation has been observed in semisolid culture with either PHA-LCM, PHA-TCM, PHA plus CSA, CSA alone, T type hairy cell-conditioned medium or human bladder carcinoma cell line-conditioned medium

(HTB9-CM).⁴² Nothing is known at present about the kind of activity that in the various conditioned media stimulates L-CFUs and supports the cell lineage-specific differentiation of L-CFUs. Hoang and co-workers recently separated leukemic blast growth factor (LBGF) from BPA and possibly from GM-CSF by Sephadex G100 fractionation of HTB9-CM.⁴² Our observation of EPO dependent leukemic colony formation suggests that factor(s) requirement of L-CFU for

in vitro growth is considerably different in different types of acute leukemia.

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Sensitivity to 5-Azacytidine of Blast Progenitors in Acute Myeloblastic Leukemia

By Chen Wang and E.A. McCulloch

In a previous study, we showed that the blast stem cells of acute myeloblastic leukemia (AML) were more sensitive to cytosine arabinoside (ara-C) when growing in suspension culture than during colony formation in methylcellulose. We suggested that the difference might be explained by considering the cellular mechanisms responsible for growth in suspension and colony formation. In the former, the clonogenic cells increase in number (self-renewal); in the latter, most of the divisions are terminal. The increased sensitivity to ara-C in suspension might then be attributed to its ability to inhibit self-renewal to a greater degree than cell division generally. A test of this hypothesis would be to compare the survival curves in suspension and in methylcellulose using a drug that spared or stimulated self-

renewal. Such an agent is 5-azacytidine (5-aza) and has the additional advantage that its analogue, 6-azacytidine (6-aza) has no effect on self renewal. The data supported the hypothesis, since clonogenic AML blasts were much less sensitive to 5-aza in suspension than in methylcellulose. The effect of 6-aza, while qualitatively similar, was much less marked. Controls showed that the difference in survival curves could not be explained on a kinetic basis or by the secretion of growth factors by 5-aza-treated cells. We suggest that a comparison of the effects of drugs in suspension and in methylcellulose may be useful in preclinical screening of putative anti-AML compounds.

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SELF-RENEWAL, the process through which stem cells give rise to daughters with sustained proliferative potential, is essential for the maintenance of normal or malignant hemopoietic clones. The blast progenitors in acute myeloblastic leukemia (AML) have been shown to be capable of self-renewal.¹ These malignant stem cells form colonies in methylcellulose-containing cultures when stimulated by appropriate growth factors.² A minority of cells from blast colonies form new blast colonies on replating.¹ Furthermore, clonogenic blast cells increase exponentially in suspension culture.³ Both these events can be measured quantitatively, and each may be considered a manifestation of blast self-renewal.

We suggested that self-renewal may be an important target for therapeutic agents used in the treatment of AML.⁴ First, there is a consistent association between high self-renewal capacity as measured by replating pooled blast colonies, (secondary plating efficiency, or PE2) and poor response to treatment.^{5,6} Second, we showed that cytosine arabinoside (ara-C), a drug included in most effective anti-AML regimens, decreases self-renewal of blast progenitors cultured in its presence.^{4,7} Recently, we found that ara-C is a more potent inhibitor of blast growth in suspension than of blast colony formation in methylcellulose.⁸ Moreover, preliminary data from a small number of patients provided evidence that the sensitivities of blasts measured in suspension were associated with outcome of treatment with ara-C, whereas similar survival curves obtained with the methylcellulose assay showed no such correlation.⁸

We postulated that ara-C appeared to be more toxic to growth of cells in suspension than to colony formation in methylcellulose because self-renewal was the predominant cellular event in the former, whereas colony formation reflected principally postdeterministic divisions. A test of this hypothesis would be to measure survival curves under both conditions, using an agent that increased self-renewal. The drug 5-azacytidine (5-aza) is an obvious candidate; not only does exposure to it increase self-renewal, but a control compound also exists. The analogue 6-azacytidine (6-aza) is cytotoxic but, unlike 5-aza, does not affect self-renewal.⁹

This article reports the measurement of 5-aza survival curves for AML blast cells exposed to the drug either in

suspension or in methylcellulose. As predicted, growth in suspension was less sensitive to 5-aza than was colony formation in methylcellulose.

MATERIALS AND METHODS

Leukemic cells. Heparinized peripheral blood was obtained from 20 patients with AML (French-American-British, FAB, classification M1 through M5)¹⁰ at the time of diagnosis or at relapse. The diagnosis was based on morphological and clinical criteria regularly used in clinical trials of patients with AML at the Ontario Cancer Institute.^{11,12} Only patients with a high percentage of blast cells in the peripheral blood were included in the study.

Preparations of blast cells free of contaminating T lymphocytes were obtained as described previously, using two Ficoll-Hypaque density-gradient centrifugations ($P = 1.007$), the second following the formation of sheep-erythrocyte rosettes.¹³ The cells were either placed in culture at once or preserved in liquid nitrogen in the presence of 10% dimethylsulfoxide (DMSO) and 50% fetal calf serum (FCS). In some experiments, cells were tested after being maintained for up to 3 months in suspension culture.

Assay for clonogenic blast cells. The assay for clonogenic blast cells was as described previously with the exception that media conditioned by the bladder cancer cell line HTB9 (HTB9-CM) was used as a source of growth factor.^{13,14} Blast populations were cultured in 35-mm Lux dishes (Miles Laboratories, Naperville, IL) in a medium consisting of α -minimal essential medium (MEM), 20% FCS (growth medium) and 10% HTB9-CM with 0.8% methylcellulose. The cultures were incubated for 7 days in a humidified atmosphere in the presence of 5% CO₂ in air at 37 °C, after which

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time colonies containing >20 cells were counted, using an inverted microscope.

Measurement of the PE2. The secondary plating efficiency (PE2) was measured using the pooled colony technique described previously.¹ The cells were harvested from primary colonies, pooled, washed and replated in microwells at a cell density of 10^4 per well with media of the same composition used for primary cultures. Colonies with >20 cells were counted after 7-day incubation. PE2 was expressed as colonies per 10^4 cells plated.

Suspension culture method. Blast cells were cultured in suspension as described previously.³ As in the clonogenic assay, HTB9-CM was used rather than phytohemagglutinin-leukocyte conditioned medium (PHA-LCM) as a source of growth factor.¹⁴ The cells were suspended in growth medium at a concentration of 10^6 /mL in the presence of 10% HTB9-CM. After varying times of incubation, the cells were harvested and counted. Usually the cell numbers recovered at this point in the control groups were greater than the 10^6 cells/mL used at the beginning of the culture; the suspensions were washed and replated in methylcellulose for blast colony formation, as described earlier. This procedure provided a measurement of the number of clonogenic cells per dish recovered at each point of time in suspension culture. The calculation consisted of multiplying the number of cells recovered per dish (as determined before washing) by the plating efficiency in methylcellulose. In some instances, cells recovered from suspension culture were washed, resuspended at 10^6 /mL, and recultured in suspension.

Exposure to drugs. 5-Aza was obtained from Sigma Chemical, St Louis, and 6-aza (P-L Biochemicals, Milwaukee). The drugs were dissolved in phosphate-buffered saline (0.02 mol/L Na_2HPO_4 ; 0.02 mol/L NaH_2PO_4 , pH 6.5; 0.42 mol/L NaCl) just before use.¹⁵ Ara-C was obtained from Upjohn, Don Mills, Canada. Adriamycin was obtained from Adria Laboratories of Canada, Mississauga, Ontario. Cells in either methylcellulose or suspension cultures were exposed to increasing concentration of drugs in growth medium modified to exclude ribonucleosides and deoxyribonucleosides and buffered with HEPES. The survival curves were calculated by comparing the number of colonies or the number of clonogenic cells recovered at each dose with controls, cultured in the absence of drug. Simple negative exponential survival curves were obtained for blasts exposed to each of the drugs, either in suspension or in methylcellulose. These curves can be described by a single slope parameter, the dose required to reduce survival to 10% of control (D_{10}). A diagrammatic representation of the procedure for obtaining survival curves in methylcellulose and in suspension is given in ref. 8.

Stability of 5-Aza. Stability of the structure of 5-aza was determined by measuring absorption at 241 nm, using a Unicam sp 1800 spectrophotometer, as described by Notari and De Young.¹⁵ We consider this procedure satisfactory, although it does not detect the intermediate decay products that are disclosed by high-pressure liquid chromatography, since our concern was only with 5-aza in buffer rather than in a complex mixture of compounds.¹⁶

RESULTS

Figure 1 contains typical survival curves for blast stem cells maintained for 2 weeks in suspension culture and then exposed for 7 days to 5-aza, adriamycin, or ara-C, either in suspension or in methylcellulose. It is evident from Fig 1 that the comparisons of the survival curves in suspension and methylcellulose differed for the three drugs. As in previous studies,⁸ the adriamycin survival curves in suspension and methylcellulose were indistinguishable; in contrast, ara-C appeared more toxic to cells in suspension than to the same population in methylcellulose. The reverse was found for

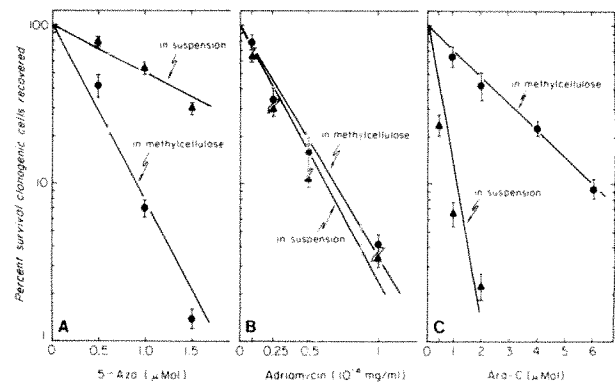


Fig 1. Survival curves for cells exposed to 5-azacytidine (5-aza) (A), adriamycin (B), or cytosine arabinoside (ara-C) (C) either in methylcellulose or in suspension. In the former instance, the drug was included in the cultures used for colony formation. In the latter instance, the drug was present during 7 days of suspension culture; the cells were then washed and tested for colony formation in methylcellulose. The number of clonogenic cells in each culture was calculated from the number of nucleated cells recovered from the 7-day cultures and plating efficiency (described in text). The cells used for this experiment were maintained in suspension culture for 2 weeks. Error bars show SD. Survival curves were drawn by linear regression. This procedure yielded two curves for the experiment with adriamycin, but SD of the points overlap and the two curves are not different.

5-aza; for this drug, the D_{10} value in suspension was much greater than the D_{10} in methylcellulose. Similar data were obtained when cells were exposed to the three drugs after 3 months in suspension culture. Table 1 contains the data from 20 experiments comparing 5-aza survival curves for blasts obtained either freshly or after cryopreservation, as well as the D_{10} values in suspension and methylcellulose. Table 1 also shows the French-American-British (FAB) classification, the blasts in the peripheral blood, and the PE2 values for each patient. It is evident that the D_{10} values in suspension were always greater than the D_{10} values in methylcellulose ($P < .00001$); furthermore, there was no correlation between the two D_{10} measurements ($r = -.217$). In agreement with previous findings,⁹ colonies in methylcellulose surviving exposure to 5-aza usually had increased values of PE2. In 4 of the 20 examples, however, decreases were seen. As in previous experiments,⁹ treatment with 5-aza did not stimulate secondary colony formation if it was absent in controls (two examples in Table 1).

Controls. A number of controls were required to establish the validity of the differences in D_{10} illustrated in Table 1. First, the construction of survival curves depends on a linear relationship between the number of cells placed in culture and the growth observed at the end of the assay. Linearity has been shown for methylcellulose cultures; for the suspension assay, a linear relationship exists between the number of nucleated cells used to initiate the cultures and the number of clonogenic cells recovered from each dish after 7 days.³ It was still possible, however, that treatment with 5-aza might change this relationship. Figure 2 shows the results of testing for linearity both in control suspension cultures and in cultures containing 5-aza; linearity was maintained under both conditions.

Table 1. Clinical Characteristics and Response of Blast Cells to 5-Aza of Patients With AML

Patient No.	Diagnosis (FAB)	Peripheral Blood				D ₁₀ (μmol/L)		PE2*	
		Hemoglobin (g/dL)	Platelets (× 10 ⁹ /L)	WBC (× 10 ⁹ /L)	Blasts (%)	M.C.	Suspension	Control	5-Aza
1	M2	6.7	17	39	58	0.75	3.5	51	85
2	M4	11.5	121	203	78	1.0	2.6	154	99
3	M5	9.8	150	35	29	1.04	3.1	324	362
4	M5	13.7	185	3.6	21	1.1	3.12	8.6	14
5	M4	11.9	271	39	77	1.2	3.2	1439	1749
6	M1	7.7	6	11.6	83	0.7	5.2	235	417
7	M4	10.8	290	43	76	1.2	2.8	10	24
8	M4	11.9	101	24	87	0.9	3.9	5.3	11
9	M2	9.4	37	178	94	NA	2.2	0	0
10	M5	8.4	62	75	19	1.1	2.8	168	487
11	M1	11.7	47	5.5	28	1.2	2.0	112	86
12	M3	10.1	54	12	54	0.6	2.5	123	165
13	M1	9.2	38	66	94	0.8	1.1	0	0
14	M2	8.7	34	29.7	42	0.9	NA	0	0
15	M4	8.5	76	47	29	1.0	2.0	42	58
16	M4	9.6	17	11.9	85	1.0	1.6	283	241
17	M4	11.6	94	90.7	87	0.82	1.7	20	15
18	M4	9.7	58	34	68	0.8	4.8	3.7	7
19	M4	12.4	70	79	20	0.92	3.5	6.5	30
20	M2	11.0	35	41	85	0.95	1.1	356	420

5-Aza, 5-azacytidine; FAB, French-American-British classification; D₁₀, dose required to reduce survival to 10% of control; PE₂, secondary plating efficiency; M.C., methylcellulose culture.

*PE₂ values are the mean of four replicate microwells with 1×10^4 cells from primary methylcellulose culture. The 5-aza group was at a concentration of 1.0 μmol/L.

Second, the outcome of suspension cultures is more dependent than colony formation on growth kinetics; in the latter, the number of cell divisions occurring during the assay period is reflected in colony size and does not enter into the analysis unless the number of cells in each colony is reduced below the counting criterion of 20 cells to a colony. For the suspension method, however, the number of doublings in 7 days contributes directly to the number of clonogenic cells recovered in each dish. Controls were necessary, therefore, to test the

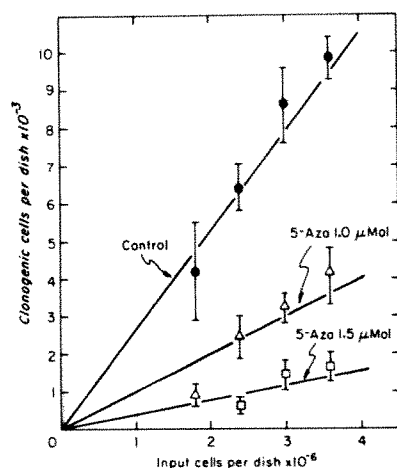


Fig 2. The linear relationship between number of nucleated cells added to suspension cultures and recovery of clonogenic cells after 7 days. Linearity is maintained in the presence of 5-azacytidine (5-aza). Error bars show SD; lines were drawn by linear regression.

possibility that the apparent difference in the survival curves in suspension and methylcellulose could be explained by growth kinetics in suspension. Blast cells were grown in suspension in the presence or absence of 5-aza at a concentration of 10^{-6} mol/L, a concentration that is close to the D₁₀ value in methylcellulose (Table 1). Two examples are illustrated in Fig 3. As anticipated, there was a sharp drop in clonogenic cells in each dish in the presence of drugs as compared with control. Suppression persisted for ~3 days and then growth resumed, with kinetics similar to the controls.

To exclude an early effect of 5-aza similar to that which occurred with ara-C, a detailed survival curve was prepared for cells maintained for >1 year in suspension and then exposed to 5-aza for 8 hours; the cells were then washed twice and either placed in suspension or plated in methylcellulose. The cells cultured in suspension were plated in methylcellulose after 7 days to obtain a survival curve in the usual manner. Figure 4 depicts the survival curves from this experiment, using the same cells, exposed to 5-aza briefly and then either plated directly in methylcellulose or maintained for 7 days in suspension. With this design, the reduction in growth in suspension was much less than that which occurred in methylcellulose.

Complete survival curves were also prepared for blast populations cultured for 3 days with or without 5-aza and then plated in methylcellulose to measure survival. Blasts exposed to 5-aza for 7 days in suspension or in methylcellulose were included in the same experiments as positive controls. Results from one of two similar experiments are shown in Fig 5. As in the curves prepared from cells exposed

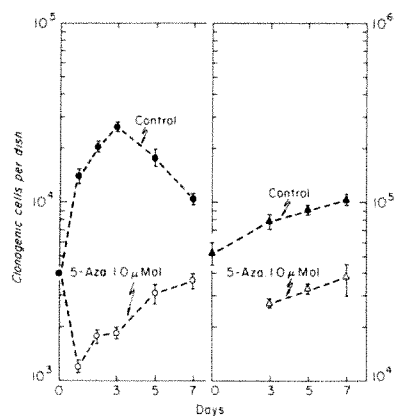


Fig 3. Kinetics of blast clonogenic cells in suspension culture in the presence or absence of 5-azacytidine (5-aza). Error bars are SD. Two examples are shown.

to drug for 8 hours (Fig 4) or 7 days, the clonogenic cells exposed in suspension culture for 3 days were more resistant than in methylcellulose.

We also assessed the survival of the 5-aza structure. The drug was incubated in buffer without cells, using spectrophotometric absorption at 241 nm,¹⁵ as described in the Materials and Methods section. The drug was unstable, approaching undetectable levels after 3 days, a time consistent with the period of suppression of growth in culture.

As a third control, the effects of 6-aza were compared with those of 5-aza; survival curves were measured in seven experiments, both in suspension and methylcellulose. In agreement with the previous data,⁹ 6-aza was less toxic than 5-aza, using the clonogenic assay ($P = .04$). In contrast, in suspension, 6-aza was a more effective inhibitor than 5-aza ($P = .04$), although the D_{10} values in suspension for 6-aza were greater than the D_{10} values in methylcellulose ($P = .004$). For this subset of the data on blasts exposed to 5-aza, as for the whole data set, the D_{10} values for survival curves measured in suspension were much greater than those obtained using the methylcellulose assay ($P = .007$). Furthermore, the difference between survival curves in methylcellulose and in suspension for 5-aza was significantly greater than the differences for 6-aza ($P = .011$). Representative survival curves illustrating these features are shown in Fig 6.

Growth factor production. 5-Aza-treated blasts might show increased self-renewal as compared with controls if

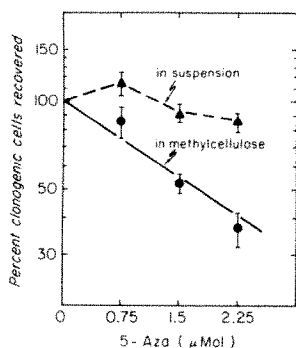
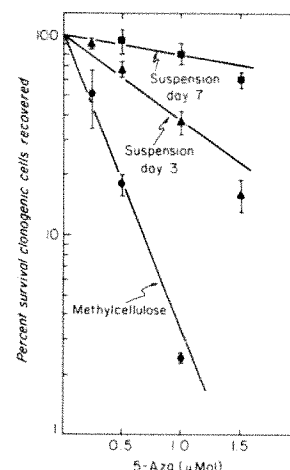


Fig 4. Survival curves for blast progenitors exposed to 5-azacytidine (5-aza) for 8 hours in suspension and then cultured for 7 days either in suspension or in methylcellulose. Cells used in this experiment had been maintained for >1 year in suspension culture. Error bars are SD.

Fig 5. Representative survival curves for blast progenitors exposed to 5-azacytidine (5-aza) in suspension for 3 or 7 days compared with a survival curve for cells exposed to drug for 7 days in methylcellulose. Error bars are SD.



they were induced by the drug to secrete growth factors. Previously, we tested for this possibility, using cells that produced small amounts of growth factor; no increase was found following exposure to 5-aza.⁹ We extended these observations to cells that required HTB9-CM for growth and another blast population, representative of a rarer phenotype, in which good growth occurred in the absence of added HTB9-CM. For the former, it was necessary to add HTB9-CM to the cultures; therefore, controls were needed for the carryover of growth factors when the supernatants were tested for their effects on blast growth. This required titrations of HTB9-CM that had been incubated for 7 days in the presence of growth medium but without cells. For the population capable of sustained growth, these further controls were not required since these cells could be maintained in culture in the presence or absence of 5-aza without added growth factors. The results are given in Fig 7. All activity that occurred in cultures of cells requiring growth factor could be attributed to carryover of HTB9-CM; furthermore, no increase in growth-promoting ability was observed when

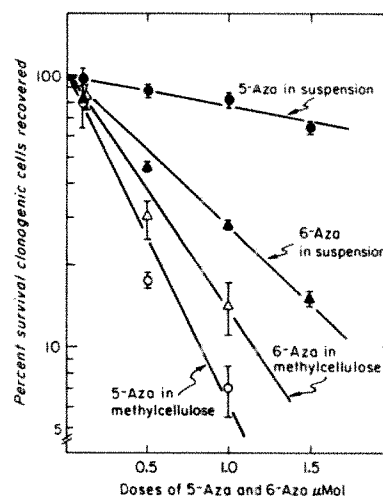


Fig 6. Representative survival curves for blast progenitors exposed to 5-azacytidine (5-aza) or 6-azacytidine (6-aza) in suspension or in methylcellulose. Lines were drawn by linear regression.

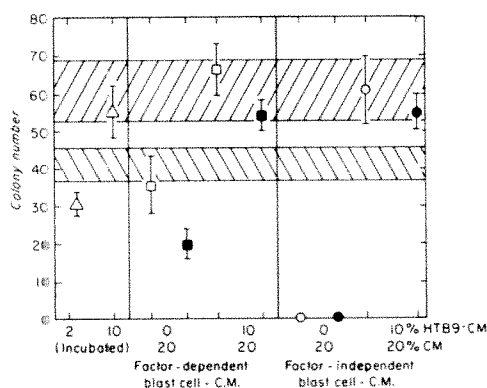


Fig 7. Experiments designed to show whether blast populations secreted growth factors active on blast cells. The top hatched area is the 95% confidence interval for blast cells exposed to 10% HTB9-conditioned medium (HTB9-CM). The lower hatched area is the 95% confidence interval for blasts exposed to 2% HTB9-CM, the amount calculated as present in the media of blast cells 7 days after culture, in which the cultures were initiated with 10% HTB9-CM. Colony number stimulated by the addition of conditioned media is shown on the vertical axis. The percentage and source of conditioned media under test is shown along the horizontal axis. Triangles (left panel) show the number of colonies stimulated by 2% or 10% HTB9-CM incubated for 7 days in the absence of cells. The activity was stable under these conditions. In the center and right-hand panels, open symbols represent results of supernatants from blast cells cultured in the absence of 5-azacytidine (5-aza); solid symbols represent supernatants of cultures in the presence of the drug. The middle panel (square symbols) shows results using cells that required HTB9-CM for growth; 20% conditioned media from these cells were less effective than the control values for HTB9-CM incubated for 7 days. Addition of 10% HTB9-CM to the blast supernatant yielded an activity equivalent to 10% HTB9-CM alone. The right panel (circles) shows data from cultures of cells that grew well without the addition of HTB9-CM. Supernatants from these cultures, with or without 5-aza, failed to stimulate blast colony formation. However, addition of 10% HTB9-CM gave the anticipated activity. Thus, blast cells in suspension did not secrete growth factors, nor did they when inhibitors were present.

5-aza was added at the time of culture initiation. Cells that grew without added HTB9-CM did not secrete blast growth factors when cultured for 7 days either with or without 5-aza. Controls in which HTB9-CM was added to supernatants from blast cultures were effective stimulators, indicating that residual cytotoxic drug or other inhibitors were not demonstrated in the supernatants.

Repeated exposure to 5-aza. The effects of 5-aza on blast progenitor self-renewal made it reasonable to question whether one more exposure to the drug would prolong the time for which blast progenitors could be maintained in suspension. Figure 8 shows the results of an experiment in which control and 5-aza-treated blast progenitors were maintained in suspension culture for 56 days. As in previous experiments of this design, the cells were harvested at weekly intervals, counted, washed, and recultured at $10^6/\text{mL}$. The data are presented as a cumulative growth curve, calculated on the basis of the changes occurring in each weekly culture period. One experimental group was exposed to 5-aza (0.5×10^{-6} mol/L) during the first week only; a second group was treated a second time between days 21 and 28. As

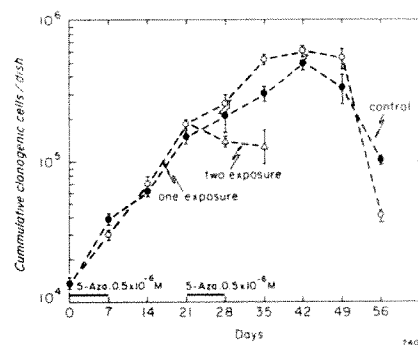


Fig 8. Kinetics of blast cell growth in controls and cultures exposed once or twice to 5-azacytidine (5-aza) at a concentration of 0.5×10^{-6} mol/L.

anticipated from the 5-aza survival curves for blasts in suspension, the single drug exposure led to a slight decrease in clonogenic cells after 7 days. Thereafter, the treated group contained more progenitors than did the control for 49 days; both then declined rapidly. The second drug treatment led to an immediate cessation of growth; the cultures could be maintained through only one subsequent passage. In this and three similar experiments, exposure to 5-aza did not prolong time in culture. Furthermore, the cytotoxic effects of a second drug treatment were much greater than at the first exposure.

DISCUSSION

The work described in this paper was undertaken to test and extend our previous observation that blast cells were more sensitive to ara-C when exposed in suspension than in methylcellulose culture. We proposed that this difference might be explained by findings indicating that ara-C was a more effective inhibitor of blast cell renewal than of terminal divisions.⁸ Thus considered, increased sensitivity in suspension reflected the predominant role of renewal in this assay whereas the terminal divisions occurring in methylcellulose were less affected by the drug. This hypothesis led to the prediction that a cytotoxic agent that increased the probability of renewal in surviving stem cells would be less toxic in suspension than in methylcellulose. 5-Aza has been shown to increase PE2 and was chosen for the test. Figure 1 shows that three classes of comparisons may be found when survival curves are measured for the same cells in suspension and methylcellulose. For adriamycin, an agent that is equally toxic for renewal and terminal divisions, the survival curves in suspension and methylcellulose were indistinguishable. As noted previously,⁸ ara-C was more sensitive in suspension than in methylcellulose. As predicted by the hypothesis, the findings with 5-aza were the reverse of those with ara-C; that is, the 5-aza D_{10} was greater for blasts exposed in suspension than for the same populations cultured with the drug in methylcellulose.

The validity of survival curves obtained using either the clonogenic assay in methylcellulose or growth in suspension is dependent on the linear relationship between input cells and the manifestations of growth after 7 days; however, when results obtained with the two assays are compared, the cellular events leading to the endpoints for each must be

considered. Colony formation in methylcellulose requires that a progenitor divide often enough to have at least 20 progeny. The natures of the divisions, renewing or terminal, do not enter into the assessment; neither is there added weight for large colonies. A kinetic component of the methylcellulose assay might emerge if the times of incubation were prolonged; however, this is not practical experimentally, since colonies usually disintegrate later.¹ In contrast, the assessment of the suspension assay reflects the number of renewing divisions occurring in 7 days; consequently, the measurement is very kinetics-dependent. The significance is apparent from the kinetic data presented in Fig 5, in which it is evident that the number of clonogenic cells recovered 7 days after exposure to 5-aza depends on both the inhibitory effects of drug during the 3 days it remains active and the subsequent recovery. A comparable recovery in colony formation in methylcellulose is unlikely because, as noted earlier, the colonies disintegrate after 7 to 8 days in culture and the number of clonogenic cells that can be recovered from the dishes also decreases markedly.¹ These findings are compatible with the postulate that conditions in methylcellulose are less favorable for growth than are conditions in suspension.

We chose to retain the 7-day interval for both assays as the basis for comparisons between them, even though this allows time for recovery in the suspension assay. The advantage is that regrowth of malignant stem cells after chemotherapy, a clinically important function, is retained. Studies in which the cells were exposed briefly to 5-aza in suspension and then either cultured in suspension or methylcellulose for 7 days also showed, however, that the progenitors were more resistant in suspension than in methylcellulose. Other experiments, in which blasts were exposed in suspension for 3 days and then plated in methylcellulose gave comparable results. Thus, it is improbable that the findings with 5-aza can be explained by the kinetics of recovery of drug-exposed cells.

The mechanisms underlying the generation of different survival curves in suspension and methylcellulose remain to be determined. We excluded drug-induced secretion of growth factors following treatment of cultured cells with either ara-C⁹ or 5-aza (Fig 7). Selection in suspension of a sensitive or resistant population of blast progenitors might

provide an explanation of the data. The results presented in Fig 1 argue against this possibility; the experiments were performed on cells that had been maintained in exponential growth for either 2 weeks or 3 months. Nonetheless, the comparison of survival curves observed for cells was similar to that observed with fresh samples and did not change when the time in suspension was extended.

The comparison of the effects of 5-aza and 6-aza may contribute to the explanation of the observed phenomenon (Fig 6). DNA hypomethylation is a known effect of the former but not the latter.^{17,18} In the clonogenic assay, 5-aza is more toxic than 6-aza; the reverse occurs when the suspension method is used. Thus, DNA hypomethylation may contribute to either the inhibitory effects of 5-aza in suspension or recovery from them. It should be noted that D₁₀ 6-aza values are greater in suspension than in methylcellulose, however, although the difference between the two culture conditions is significantly less than for 5-aza.

Despite its effect of self-renewal, 5-aza treatment did not facilitate the establishment of continuous lines. Instead, toxicity of the drug appeared to be cumulative, since a second exposure, 2 weeks after the first, with two intervening subcultures, led to a much greater decrease in growth than anticipated on the basis of the survival curves or response to a single exposure. The mechanism underlying these observations is unknown. It is striking, however, that blast cells, so aggressive in patients, cannot regularly be maintained indefinitely in culture. It may be that further improvements in the culture techniques are required to achieve long-term growth.

Finally, the hypothesis that differences in survival curves measured in suspension as compared with methylcellulose may reflect specific drug effects on self-renewal leads to the prediction that a correlation may be found between D₁₀ values in suspension and clinical outcome. For ara-C, preliminary evidence is available in support of the prediction.⁸ Unfortunately, the blasts used for the 5-aza experiments were obtained from patients at initial presentation and at relapse. This heterogeneity in the patient population is sufficient to prevent valid statistical analysis. More survival curves on blasts from newly diagnosed patients are now being collected.

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Purification and Partial Amino Acid Sequence of Human Platelet Membrane Glycoproteins IIb and IIIa

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The glycoprotein (GP) IIb-IIIa complex was isolated from human platelet membranes by immunoaffinity chromatography using a monoclonal antibody specific for GP IIb-IIIa. GP IIb and IIIa were further separated in the presence of sodium dodecyl sulfate (SDS) by gel filtration high-performance liquid chromatography (HPLC). Two cycles of this procedure yielded almost complete separation of homogeneous preparations of GP IIb and IIIa. Each protein was then digested with lysyl endopeptidase (*Achromobacter* protease I), which cleaves at the carboxyl side of lysine

residues, and the resulting oligopeptides from GP IIb and IIIa were fractionated with HPLC using a C18 reverse-phase column. Comparison of the elution profiles showed no obvious homology between the two proteins. Amino acid sequences of selected oligopeptides from each glycoprotein were determined using a gas-phase protein sequencer. Sixty amino acid residues (26 residues for IIb and 34 residues for IIIa) were identified.

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GLYCOPROTEINS (GP) IIb and IIIa are major constituents of the platelet plasma membrane and serve as the cellular receptor for fibrinogen.¹⁻⁹ Abnormalities of the GP IIb-IIIa complex result in failure of platelet aggregation. For example, patients with Glanzmann's thrombasthenia have either a deficiency of GP IIb-IIIa or abnormal molecules.^{9,10} In an unreduced state, GP IIb and GP IIIa have mol wt of 140,000 and 95,000, respectively, and they are present as a Ca²⁺-dependent heterodimer complex in both solubilized platelet extracts and in the plasma membrane of intact platelets.^{11,12} Despite the similarity of the amino acid composition of GP IIb and GP IIIa,^{13,14} tyrosyl tryptic peptide maps of these proteins are dissimilar.^{13,15,16}

Murine monoclonal antibodies specific for the GP IIb-IIIa complex inhibit both fibrinogen binding and platelet aggregation.¹⁷⁻²¹ In this study, we used a newly prepared murine monoclonal antibody (designated HPL-1), which recognizes GP IIb-IIIa and inhibits platelet aggregation induced by adenosine diphosphate (ADP), collagen, and a number of other physiological agents.²²⁻²⁴ GP IIb and IIIa were copurified by immunoaffinity chromatography with HPL-1 and then separated from each other by gel filtration high-performance liquid chromatography (HPLC).²⁶ Each purified GP was digested with lysyl endopeptidase. The resulting HPLC-generated oligopeptide maps and partial

amino acid sequence analysis showed no evidence of similarity between the two proteins.

MATERIALS AND METHODS

Production and purification of a monoclonal antibody. The production and specificity of the murine monoclonal antibody HPL-1 (IgG₁) used in this study have been described in detail elsewhere.²²⁻²⁴ In brief, this hybridoma was derived from (BALB/c × C57BL/6) female mice immunized with human platelets. By immunoprecipitation experiments with ¹²⁵I-labeled platelet lysates, HPL-1 precipitated the antigen molecules of 127 and 104 kd under reduced conditions and antigen molecules of 138 and 90 kd under unreduced conditions. This antibody inhibited platelet aggregation induced by ADP or collagen and did not react with platelets of thrombasthenia patients. Thus, the antigen recognized by HPL-1 antibody was considered the GP IIb-IIIa complex. HPL-1 was purified from ascites fluid by precipitation with saturated ammonium sulfate followed by diethylaminoethanol (DEAE) cellulose column chromatography.¹⁰ The purified antibody was dialyzed against coupling buffer (borate buffer pH 8.3 containing 0.5 mol/L of NaCl).

Immunoaffinity purification of the GP IIb-IIIa complex. Ten milligrams of purified HPL-1 antibody was coupled to 2 mL of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's directions. Platelets (5×10^{10}) were washed three times in phosphate-buffered saline (PBS) pH 7.4, containing 2.5 mmol/L of EDTA; whole platelets were then solubilized in 50 mL of extraction buffer (10 mmol/L of Tris-HCl, pH 7.4, 0.15 mol/L of NaCl, 1 mmol/L of phenylmethylsulfonyl fluoride (PMSF), 2.5 mmol/L of EDTA, and 0.5% Lubrol PX) for 45 minutes at 0 to 4 °C with periodic vortexing. An additional 50 mL of the extraction buffer was added, and the sample was incubated for another 30 minutes to permit complete solubilization. The lysate was centrifuged at 100,000 g for 30 minutes at 4 °C to remove particulate matter, and the supernatants containing platelet membrane protein were pooled. The platelet lysate was first applied to a column made of bovine serum albumin (BSA)-conjugated Sepharose 4B and equilibrated in 10 mmol/L of Tris-HCl, pH 7.4, 0.15 mol/L of NaCl, and 0.5% Lubrol. The throughout fraction, from which materials adsorbed nonspecifically to Sepharose and/or BSA had been removed, was applied to a HPL-1-Sepharose 4B affinity column (2 mL) equilibrated with the same buffer as described above. The column was washed extensively with a high-salt buffer (10 mmol/L of Tris-HCl, pH 7.4, 1 mol/L of NaCl and 0.5% Lubrol), and the retained protein was eluted with 50 mmol/L of diethylamine, pH 11.5, containing 0.1% Lubrol. The eluate was immediately neutralized by the addition of 200 µL of 2 mol/L of Tris-HCl, pH 7.2, and stored until further use at -80 °C. Protein concentration was determined using the Bio-Rad (Richmond, CA) Protein Assay kit.

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 20 to 30 μ g of the purified protein was electrophoresed in an 8% polyacrylamide slab gel according to the method of Laemmli.²⁵ Reduced samples contained 5% β -mercaptoethanol. Gels were stained with Coomassie brilliant blue. The following proteins were electrophoresed as mol wt standards: *Escherichia coli* RNA polymerase containing subunits of β' (160,000), β (150,000), σ (90,000), α (40,000); BSA (67,000).

Separation of glycoproteins IIb and IIIa by gel filtration HPLC. The affinity-purified GP IIb-IIIa complex was concentrated by lyophilization and then redissolved in water containing 1 mmol/L of EDTA and 0.1% SDS. The mixture was warmed for 60 minutes at 37 °C to achieve complete solubilization of protein complexes. To dissociate and purify the individual glycoproteins, the sample was applied to HPLC (Spectra-Physics, San Jose, CA) using a 60-cm TSK 4000SW gel filtration column equilibrated in 50 mmol/L of Tris-HCl, pH 7.2, 1 mmol/L of EDTA, and 0.1% SDS as described by Newman and colleagues.²⁶ Chromatography was performed at a flow rate of 0.5 mL/min, and the protein was monitored by measuring the absorbance at 280 nm. Peak fractions containing GP IIb or GP IIIa were collected separately. Each fraction was rechromatographed under conditions of the first chromatography to achieve homogeneous preparations of each GP. Fractions were pooled and lyophilized.

Removal of SDS from GP IIb and GP IIIa preparations after gel filtration HPLC. Although nonionic detergents such as NP-40 or Lubrol do not inhibit proteolytic digestion, SDS affects the proteinase activity of lysyl endopeptidase. Therefore, removal of SDS from protein samples was necessary prior to enzymatic digestion of GP IIb and GP IIIa. To achieve complete extraction of SDS, we used the method described by Henderson and co-workers.²⁷ In brief, the extraction solvent was a freshly prepared mixture of anhydrous acetone:triethylamine:acetic acid:water (85:5:5:5; vol/vol/vol/vol). Lyophilized GP IIb and GP IIIa samples containing SDS were dissolved directly in 1 mL of the extraction solvent containing 1 μ g of lysyl endopeptidase.²⁸⁻³⁰ The solution was kept at -20 °C for at least 1 hour. The proteins were precipitated by centrifugation (5,500 g for 45 minutes at -5 °C). The enzyme and the substrate were coprecipitated in this way to give a subsequent more reproducible digestion.³¹ To insure complete removal of SDS, the protein precipitate was washed twice with fresh extraction solvent. Finally, the precipitate was washed twice with acetone to remove traces of the extraction solvent. Residual acetone was removed by lyophilization.

Digestion of GP IIb and GP IIIa polypeptides with lysyl endopeptidase. The SDS-free lyophilized samples were each redissolved in 500 μ L of 50 mmol/L of Tris-HCl, pH 9.2, 0.1% Lubrol. An additional 1 μ g of lysyl endopeptidase was added to each sample. The mixtures were incubated at 37 °C for 24 hours and then lyophilized. Lysyl endopeptidase (*Achromobacter* protease I, Wako, Japan) is a proteolytic enzyme from *A. lyticus* M 497-1.²⁸⁻³⁰ This enzyme hydrolyzes peptide bonds at the carboxyl side of lysine residues in a manner similar to that of trypsin. This enzyme attacks the Lys-Pro bond, which is resistant to trypsin, and does not attack the arginyl bond, which is easily hydrolyzed by trypsin. Moreover, this enzyme also cleaves aminoethyl cysteine-X bonds.

Purification and sequencing of oligopeptides digested with lysyl endopeptidase. Oligopeptides were fractionated by HPLC on a 4 $\times$ 250-mm reverse-phase (RP) column (Synchropak RPP C18 column, pore size 300 Å, Synchrom, Linden, IN). The peptides were eluted with a linear gradient of 0% to 45% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.7 mL/min for 120 minutes. Absorbance at 214 nm was monitored, and peaks were collected manually. Fractions obtained in this manner were not rechromatographed because they were already pure for subsequent analysis even after one cycle of chromatography. Oligopeptides

eluted as sharp peaks from RP-HPLC were selected as samples for amino acid sequencing because broad peaks occasionally contained two oligopeptides. Selected samples were lyophilized, and their amino acid sequences were determined on an Applied Biosystems (Foster, CA) Model 470A protein sequencer according to published procedures.^{32,33} Phenylthiohydantoin (PTH) amino acids were analyzed by RP-HPLC.

RESULTS

Isolation of the platelet membrane GP IIb-IIIa complex. The GP IIb-IIIa complex was purified from solubilized human platelets by chromatography on HPL-1-Sepharose 4B. Typical yields of the GP IIb-IIIa complex were ~500 μ g/10¹⁰ platelets. SDS-PAGE analysis of the immunoaffinity purified materials is shown in Fig 1A. The apparent mol wt of isolated GP IIb under unreduced and reduced conditions was 137,000 and 116,000, respectively; that of isolated GP IIIa under unreduced and reduced conditions was 90,000 and 100,000, respectively. These variations of apparent sizes of GP IIb and IIIa are in agreement with their previously reported behaviors.^{10,20} Densitometry scans of Coomassie brilliant blue-stained gels showed that GP IIb and IIIa at this stage were ~80% pure.

Separation of GP IIb and GP IIIa proteins. Although GP IIb and IIIa form a calcium-dependent heterodimer complex even in the presence of nonionic detergents, they can be dissociated with SDS and EDTA. Therefore, gel filtration HPLC was performed using a solution containing 0.1% SDS and 1 mmol/L of EDTA. As seen in Fig 2A, two GPs were well separated under the conditions used. Each protein peak was collected separately and rechromatographed to achieve complete purification (Fig 2B and C). SDS-PAGE indicated that the two proteins were separated from each other under these conditions and purified to apparent homogeneity (Fig 1B).

Proteolytic digestion of the GP IIb and GP IIIa polypeptides. After removal of SDS by solvent extraction, GP IIb and GP IIIa were digested with lysyl endopeptidase. The resulting oligopeptides were fractionated by RP-HPLC, and the peak fractions identified by ultraviolet (UV) absorbance were collected for subsequent amino acid sequence analysis. Repeated analysis of the peptide mapping gave the same result. As shown in Fig 3, the oligopeptide maps of these two glycoproteins appeared to be clearly different, confirming and extending previous observations^{13,15,16} of structural differences between GP IIb and GP IIIa.

Amino acid sequence analysis. The N-terminal amino acid sequences of several oligopeptides were determined using a gas-phase protein sequencer. The results are shown in Table 1. We obtained amino acid sequences identical to those of oligopeptides IIIa-3 that had been prepared independently. Therefore, we consider our analytical system reliable. Sixty amino acid residues (26 residues for IIb and 34 residues for IIIa) were identified.

DISCUSSION

During the last several years, much evidence derived from a number of laboratories³⁻⁸ has demonstrated that the human platelet GP IIb-IIIa complex serves as the receptor for

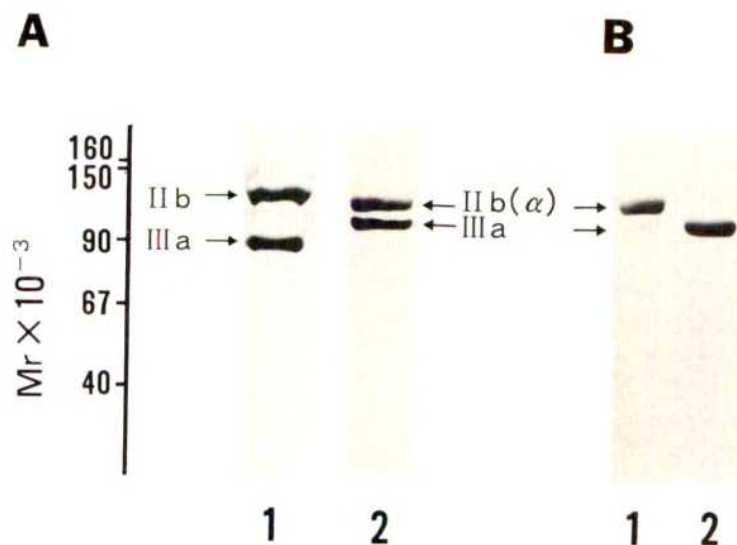


Fig 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of human platelet membrane glycoproteins GP IIb and GP IIIa. Electrophoresis was performed with 8% polyacrylamide slab gel. Gels were stained with Coomassie brilliant blue. Samples used were: (A) protein eluate from HPL-1 monoclonal antibody-coupled Sepharose 4B, unreduced (lane 1) and reduced (lane 2); (B) separated GP IIb (lane 1, reduced) and GP IIIa (lane 2, reduced) after two cycles of gel filtration high-performance liquid chromatography as described in Fig 2.

extracellular fibrinogen, a plasma protein required for platelet aggregation. GP IIb consists of two disulfide-linked subunits, GP IIb α (mol wt 116,000) and GP IIb β (mol wt 23,000). In contrast, GP IIIa consists of a single polypeptide chain with multiple intrachain disulfide bonds (mol wt 105,000 as determined from SDS gels).³⁴

Several different procedures for purifying the mixture of the two GPs have been reported. Copurification of these GPs has been reported by McEver and co-workers,¹⁰ using a monoclonal antibody affinity column; by Leung and co-workers,¹⁵ using *Lens culinaris* lectin affinity chromatography; and by Jennings and Phillips,¹⁴ using sucrose density

gradient centrifugation and Sephacryl S-300 gel filtration chromatography. The determination of the amino acid sequence of a polypeptide requires a large quantity of highly purified protein samples. Immunoaffinity chromatography using specific monoclonal antibodies is often well suited for the initial stages of purification. As shown in Fig 1A, however, the immunoaffinity purified GP IIb-IIIa complex was contaminated with a small amount of actin, which may interact with GP IIb-IIIa.³⁵ Furthermore, antibodies that are directed against only one component of the GP IIb-IIIa complex can copurify both glycoproteins.¹⁰ Therefore, fur-

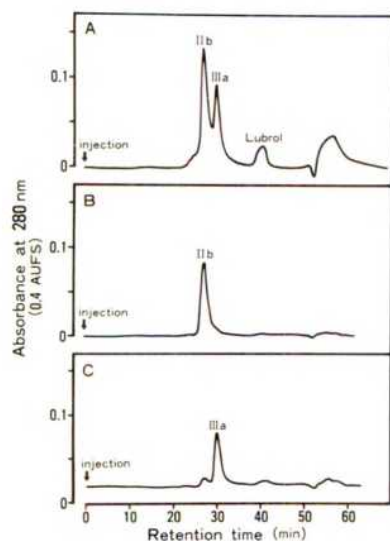


Fig 2. Separation of GP IIb from GP IIIa by gel filtration high-performance liquid chromatography. Sample was applied to a TSK-4000SW gel filtration column and chromatographed in the presence of sodium dodecyl sulfate (SDS) and EDTA at a flow rate of 0.5 mL/min. Absorbance was monitored at 280 nm, attenuated to 0.4 absorbance units full scale. (A) The first cycle chromatography of GP IIb-IIIa sample contained ~400 μ g of the protein. (B) Rechromatography of the GP IIb fraction of the first cycle. (C) Rechromatography of the GP IIIa fraction of the first cycle.

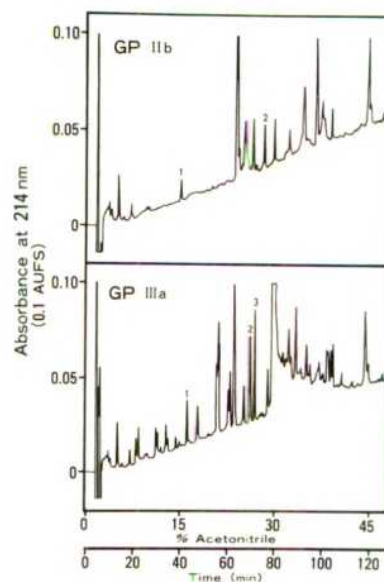


Fig 3. Reverse-phase high-performance liquid chromatography separation of the lysyl endopeptidase digested glycoproteins GP IIb and GP IIIa. As described in the Materials and Methods section, each glycoprotein was treated with lysyl endopeptidase and the resulting oligopeptides were chromatographed. Absorbance was monitored at 214 nm, attenuated to 0.1 absorbance units full scale. The oligopeptides indicated by the numbers IIb-1, IIb-2, IIIa-1, IIIa-2, and IIIa-3 were used for amino acid sequencing, the results of which are shown in Table 1.

Table 1. Amino Acid Sequences of Oligopeptides From Human Platelet Membrane Glycoproteins IIb and IIIa

Cycle	IIb-1		IIb-2		IIIa-1		IIIa-2		IIIa-3	
	Amino Acid	Yield (pmol)	Amino Acid	Yield (pmol)	Amino Acid	Yield (pmol)	Amino Acid	Yield (pmol)	Amino Acid*	Yield (pmol)
1	X	—	Ala	173	Ser†	130	His	54	Asp	66 161
2	Asn	33	Ser†	74	Phe	200	Val	158	Asp	60 203
3	X	—	Val	114	Thr†	15	Leu	196	Leu	152 233
4	Pro	55	Gln	94	Ile	116	Thr†	13	Trp	42 29
5	X	—	Leu	105	Lys	52	Leu	174	Ser†	14 50
6	Pro	48	Leu	98	—	—	Thr†	9	Ile	77 149
7	Leu	33	Val	70	—	—	Asp	60	Gln	59 128
8	Glu	34	Gln	52	—	—	Gln	69	Asn	50 131
9	Glu	40	Asp	38	—	—	Val	63	Leu	47 125
10	Asp	8	Ser†	23	—	—	Thr†	5	Gly	33 103
11	Asp	10	Leu	50	—	—	Arg	29	Thr†	5 47
12	Glu	15	Asn	24	—	—	Phe	47	Lys	12 47
13	Glu	21	Pro	23	—	—	Asn	38	—	—
14	Gly	5	Ala	39	—	—	Glu	33	—	—
15	—	—	Val	24	—	—	Glu	49	—	—
16	—	—	—	—	—	—	Val	36	—	—
17	—	—	—	—	—	—	Lys	11	—	—

(1) and (2) indicate experimental number.

X, not identified.

*As in the case of IIIa-3, repeated analysis gave the same conclusion.

†Analysis for serine and threonine could not be accurately measured due to the presence of multiple peaks obtained during analysis of the phenylthiohydantoin (PTH) amino acids. Some of those peaks are known to reflect derivatives from serine and threonine, which were specifically detected as peaks at the different positions from other PTH amino acids.

ther separation and purification are necessary for the structural analysis of these components.

We evaluated several different protocols for the separation of GP IIb from GP IIIa, including electrophoretic elution from polyacrylamide gel slices containing GP IIb or GP IIIa,¹⁵ proteolytic digestion of proteins within gel pieces¹⁶ and, finally, gel filtration HPLC.²⁶ Although electroelution or proteolytic digestion of gel slices resulted in relatively low protein yields, two cycles of gel filtration HPLC achieved both good separation of GP IIb from GP IIIa and high protein recovery. The purity of the proteins isolated by this technique provided us with the opportunity to obtain large amounts of these two membrane glycoproteins for further study.

Because small amounts of SDS in the purified glycoprotein samples interfered significantly with subsequent proteolytic digestion, we removed SDS by a method of ion-pair extraction²⁷ that resulted in reproducible digestion. Because removal of SDS from a sample may cause protein aggregation, Lubrol was added to the sample to prevent aggregation.

Nonionic detergents such as NP-40, Lubrol, etc, did not affect the enzymatic digestion.

Computerized analysis of the autoradiograms of the two-dimensional tryptic ¹²⁵I peptide maps has indicated structural differences between GP IIb and GP IIIa.^{15,16} McEver and colleagues¹³ also demonstrated similar findings when comparing HPLC tryptic peptide maps of a mixture of ¹²⁵I-IIb and ¹³¹I-IIIa. These findings are in agreement with the peptide maps of the individual glycoproteins obtained following digestion with lysyl endopeptidase. Because we did not use radioiodinated glycoproteins in these experiments, our results represent information about all oligopeptides, including those that contain no tyrosine residues. Purified oligopeptides were then successfully used as samples for amino acid sequencing. Together, our results are consistent with the hypothesis that GP IIb and GP IIIa are coded for by different structural genes that are coordinately expressed.

Amino acid sequences of the five peptides obtained in this study were compared with 7,687 protein sequences in the Protein Research Foundation/Sequence Data Base (PRF/SEQDB)³⁶ to check for homology with other proteins. The computer search against all known protein sequences failed to reveal any significant similarity to the peptides derived from either GP IIb or GP IIIa.

The isolation of cDNA clones encoding GP IIb and GP IIIa should provide important additional insights about the structure of the human platelet fibrinogen receptor and the mechanism of regulation of its expression. A recent report documents the isolation of a λ phage containing a 20-kilobase (kb) genomic library, which, on transfection, leads to the expression of the α chain of the OKM-1, LFA-1, and platelet GP IIb-IIIa molecules.³⁷ Recently, a number of investigators were also successful in the isolation of mRNA species that encode human platelet GP IIb and IIIa.³⁸ Studies are in progress in our laboratory to isolate mRNA from a megakaryoblastic leukemia cell line that expresses these glycoproteins and to establish a cDNA library. Several amino acid sequences obtained in this study have regions suitable for the synthesis of oligonucleotide probes. Probes such as these should be useful for the screening of this cDNA library.

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Prothrombin Tokushima: Characterization of Dysfunctional Thrombin Derived From a Variant of Human Prothrombin

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A mutant prothrombin, designated prothrombin Tokushima, was purified from plasma of a proband with 12% of normal plasma clotting activity and 42% of normal prothrombin antigen. The purified preparation gave a single band with the same mobility as that of "prothrombin" by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The factor Xa-catalyzed proteolysis of prothrombin Tokushima examined by SDS-PAGE was found to be identical to that of "prothrombin." Subsequently thrombin Tokushima was prepared by CM-Sepharose CL-6B column chromatography after prothrombin activation by factor Xa. The molecular weight of thrombin Tokushima estimated by SDS-PAGE was identical to that of "thrombin." Thrombin Tokushima exhibited less than 22% of normal clotting activity, and the value of k_{cat}/K_m ($\mu\text{mol}/$

$\text{L}^{-1} \text{second}^{-1}$) was less than one tenth of that of "thrombin" when Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide was used as a substrate. However, active site titration using *p*-nitrophenyl-*p*'-guanidinobenzoate failed to detect any difference between the two. Thrombin Tokushima was 2.5% as effective as "thrombin" in inducing platelet aggregation. Interaction of thrombin Tokushima with antithrombin III was much slower than "thrombin" when followed by SDS-PAGE. Based on the residual thrombin activity, it was 33% as effective as "thrombin" in forming a complex with antithrombin III. These results indicate that the molecular defect resides in the thrombin portion of prothrombin Tokushima and that the binding sites for various substrates appear to be greatly impaired.

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CONGENITAL dysprothrombinemia is a rare coagulation disorder. Only 16 variants have been reported,¹⁻¹⁷ all of them being characterized by a decrease in the functional level of prothrombin relative to antigenic level of prothrombin. Five of the prothrombin variants thus far identified have been purified and characterized. In the case of prothrombin Barcelona² and prothrombin Madrid,⁸ the functional defect has been shown to be a specific impairment of one of the two factor Xa-catalyzed cleavages, whereas in the case of prothrombin Quick,⁶ prothrombin Metz,⁹ and prothrombin Salakta,¹⁷ the defect is confined to the thrombin portion of the molecule. In 1983 we reported the first case of dysprothrombinemia in Japan as prothrombin Tokushima.^{13,14} The maternal and paternal sides have heterozygotes, respectively, for dysprothrombinemia and hypoprothrombinemia. The proband was a double heterozygote for prothrombin Tokushima and hypoprothrombinemia and therefore synthesized only the abnormal molecule. In this paper the physicochemical and enzymatic properties of prothrombin Tokushima are presented. These results demonstrate that prothrombin Tokushima is a new variant having an abnormality in the thrombin portion of the molecule.

MATERIALS AND METHODS

Blood was collected from the proband using plastic syringes that contained 1/10 volume of 3.8% trisodium citrate. Platelet-poor plasma of the proband with 12% of the clotting activity and 42% of "prothrombin" antigen was obtained after centrifugation and stored at -80°C .

Purification of prothrombin and thrombin. Prothrombin Tokushima and human "prothrombin" were purified according to the method of Morrison and Esnouf¹⁸ with slight modifications. Purified prothrombin (206 $\mu\text{g}/\text{mL}$ in 0.05 mol/L Tris-HCl, pH 8.0, containing 0.1 mol/L NaCl and 0.01 mol/L CaCl_2) was activated by bovine factor Xa (2.6 $\mu\text{g}/\text{mL}$) in the presence of bovine factor Va (28 $\mu\text{g}/\text{mL}$) and rabbit brain cephalin (100 $\mu\text{g}/\text{mL}$) for ten minutes at 37°C . Factor Xa was isolated as previously described.¹⁹ The resulting thrombin was isolated by chromatography on a CM-Sepharose CL-6B column according to Kawabata et al.²⁰ The activation mixture was applied to the column, previously equilibrated with 0.05

mol/L sodium phosphate, pH 6.5, and thrombin was eluted with a linear NaCl gradient (0 to 0.7 mol/L NaCl) in the same buffer.

Titration of thrombin with *p*-nitrophenyl-*p*'-guanidinobenzoate · HCl (*p*-NPGB). The titration of thrombin with *p*-NPGB (Nutritional Biochemicals, Cleveland, OH) was performed by the method of Chase and Shaw.²¹ Immediate burst of *p*-nitrophenol produced by the stoichiometric reaction of the catalytic site of thrombin with *p*-NPGB was monitored at 410 nm with a Hitachi 220A recording spectrophotometer (Tokyo). Thrombin active site concentration was determined using a molar extinction coefficient of 16,595 for *p*-nitrophenol at pH 8.3.

Kinetic experiments. Kinetic constants were determined using *t*-butyloxy-carbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) as the thrombin substrate (Peptide Institute, Osaka, Japan). The fluorescence of 7-amino-4-methylcoumarin (AMC) liberated from Boc-Val-Pro-Arg-MCA was monitored by a Hitachi fluorescence spectrophotometer (model 650-10S) equipped with a recorder. Emission at 440 nm was measured ($\lambda_{\text{ex}} = 380 \text{ nm}$ band pass 2 nm at 37°C). The reaction was performed at 37°C in 1 mL of 0.05 mol/L Tris-HCl (pH 8.0) containing 0.15 mol/L NaCl and 1 mmol/L CaCl_2 . The kinetic constants were calculated by the direct-linear plot based on the procedure of Cornish-Bowden.²²

Activation of factor XIII. Human placenta factor XIII was purchased from Hoechst Japan (Tokyo). The activity of factor XIII

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was measured by a modified method of the monodansylcadaverine (Sigma Chemical, St. Louis) incorporation assay.²³

Platelet aggregation. Blood was collected from a normal donor into plastic tubes containing 1/10 volume of 3.8% trisodium citrate and 25 $\mu\text{g/mL}$ apyrase (Sigma Chemical). Platelets were subsequently isolated by gel filtration on a Sepharose 2B column equilibrated with Tyrode's buffer containing 3.5 mg/mL bovine serum albumin. Aggregation studies were performed in an aggregometer (Rikadenki Kogyo, Tokyo) on 500 μL of gel-filtered platelets (10^8 cells/mL) following addition of 39 μL of thrombin solution at the final concentrations of 1.1×10^{-9} mol/L to 4.0×10^{-8} mol/L.

Interaction with antithrombin III. Purified human antithrombin III was obtained from Boehringerwerke (Marburg, West Germany). Thrombin or thrombin Tokushima was incubated at 25 °C in twofold excess relative to antithrombin III in 0.05 mol/L Tris-HCl (pH 8.0)/0.1 mol/L NaCl. Serial aliquots were removed for SDS-PAGE and for residual thrombin activity measurements. Protein concentrations were determined by absorbance measurement at 280 nm. Molecular weight (72,000) and extinction coefficient ($E_{280}^{1\%} = 13.8$) were assumed to be the same for "prothrombin" and prothrombin Tokushima. Molecular weight (36,800) and extinction coefficient ($E_{280}^{1\%} = 18.3$) were also assumed to be the same for thrombins.

SDS-PAGE (8% polyacrylamide gel) in the presence and absence of 2-mercaptoethanol was performed by the method of Weber and Osborn.²⁴ The gels were stained with Coomassie brilliant blue R-250.

RESULTS

Properties of the purified prothrombin Tokushima. Since prothrombin Tokushima was totally adsorbed onto barium citrate,^{13,14} the usual technique for prothrombin purification was employed to isolate the mutant molecule. A single peak containing prothrombin Tokushima was eluted from a DEAE-Sephadex A-50 column at the same ionic strength as that observed for "prothrombin." However, the yield of prothrombin Tokushima was about half that of "prothrombin" (data not shown). Both preparations

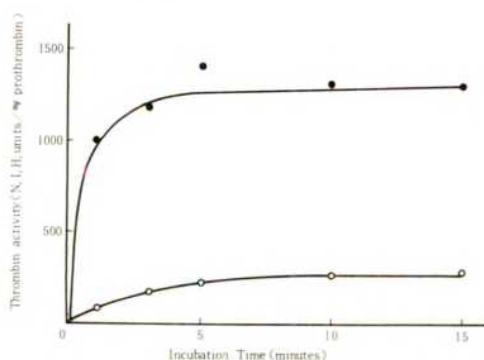


Fig 1. Generation of the thrombin activity upon activation of "prothrombin" and prothrombin Tokushima by bovine factor Xa. Sixty-two micrograms each of "prothrombin" (●) and prothrombin Tokushima (○) were incubated at 37 °C with 0.8 μg of factor Xa, 8.4 μg of bovine factor Va, and 30 μg of rabbit brain cephalin in 0.3 mL of 0.05 mol/L Tris-HCl (pH 8.0) containing 0.1 mol/L NaCl and 0.01 mol/L CaCl_2 . The generated thrombin was measured by the fibrinogen clotting assay at selected intervals until reaching a plateau. The clotting activity of α -thrombin was determined using purified human fibrinogen (approximately 95% clottable). A reference curve was prepared using NIH standard thrombin (lot B3).

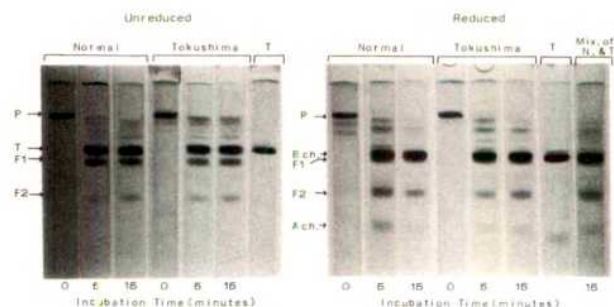


Fig 2. Factor Xa-catalyzed proteolysis of "prothrombin" and prothrombin Tokushima. The activation of each prothrombin was performed under the same conditions as described in Fig 1. After incubation at 37 °C for varying time periods, 50 μL aliquots were removed for SDS-PAGE. Prothrombin, α -thrombin, fragment 1, fragment 2, B chain, and A chain are abbreviated, respectively, as P, T, F1, F2, Bch and Ach.

migrated as a single band with the same mobility of SDS-PAGE, and their molecular weights were estimated as 72,000.

Activation of purified "prothrombin" by factor Xa in the presence of factor Va, phospholipids and Ca^{2+} produced thrombin with a specific activity of 1300 NIH U/mg after five minutes incubation. However, as shown in Fig 1, the thrombin activity produced from prothrombin Tokushima reached a plateau with about 21% of the normal level after 10 minutes.

The cleavage patterns of prothrombin by factor Xa on SDS-PAGE indicated that the proteolysis of prothrombin Tokushima was indistinguishable with that of "prothrombin," yielding thrombin, fragment 1 and fragment 2, with the molecular weights identical to those of the normal counterparts (Fig 2). Moreover, upon reduction thrombin Tokushima yielded B chain and A chain with the same molecular weight as those found in " α -thrombin." These results suggest that the cleavage sites of prothrombin Tokushima by factor Xa are normal and that the defect apparently resides in the molecule in the thrombin portion.

Preparation of the purified thrombin Tokushima. Thrombin Tokushima was prepared by CM-Sepharose CL-6B column chromatography after prothrombin activation by factor Xa, as described in Materials and Methods. As shown in Fig 3, thrombin Tokushima eluted as single peak at the same ionic strength as that observed for " α -thrombin." Figure 4 shows the SDS-PAGE patterns of thrombins thus purified. The molecular weight of the reduced and unreduced forms of thrombin Tokushima appeared indistinguishable from that of " α -thrombin."

Enzymatic properties of thrombin Tokushima. Active site titration studies using *p*-NPGb revealed that thrombin Tokushima possessed the same catalytic activity as " α -thrombin." As shown in Table 1, 95.7% of the catalytic sites in thrombin Tokushima was titratable with *p*-NPGb. On the other hand, kinetic analysis for the hydrolysis of Boc-Val-Pro-Arg-MCA by thrombin Tokushima yielded a higher K_m , a lower k_{cat} , and a lower k_{cat}/K_m ratio than that of " α -thrombin." The fibrinogen clotting activity of thrombin Tokushima was 525 NIH U/mg corresponding to 21.5% of

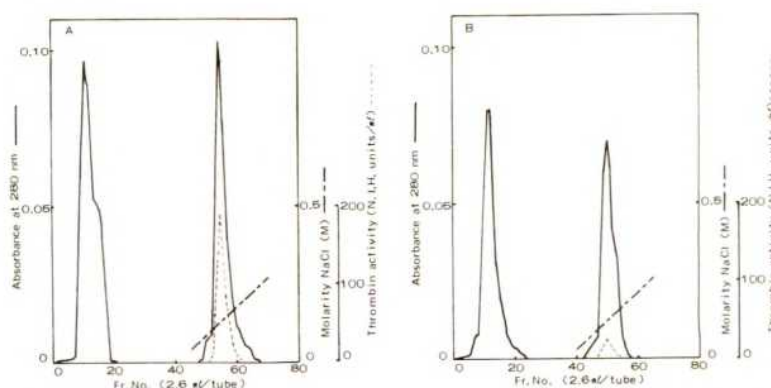


Fig. 3. CM-Sepharose CL-6B column chromatography of "α-thrombin" (A) and thrombin Tokushima (B). "Prothrombin" (1.76 mg) and prothrombin Tokushima (1.68 mg) were activated separately as described in "Methods," and the reaction mixture was applied to the column. After elution of the first peak containing fragments 1 and 2, thrombin was eluted with a 0 to 0.7 mol/L NaCl gradient in 0.05 mol/L sodium phosphate buffer, pH 6.5. Protein concentration was measured by absorbance at 280 nm (—), and the thrombin clotting activity (---) and the molarity of NaCl (·····) are shown.

normal, although it was 69.9% as effective as "α-thrombin" in the activation of factor XIII.

Figure 5 shows the thrombin-induced platelet aggregation by "α-thrombin" and thrombin Tokushima. At an enzyme concentration of 2×10^{-9} mol/L, rapid aggregation was observed with "α-thrombin," whereas no aggregation was induced by thrombin Tokushima at comparable concentration. Based on the amount of enzyme required to give an equivalent incomplete aggregation, thrombin Tokushima was less than 3% as effective as "α-thrombin."

Figure 6A shows SDS-PAGE analysis of the thrombin-antithrombin III interaction. When an excess molar ratio of "α-thrombin" to antithrombin III was employed, the band of antithrombin III disappeared within ten minutes incubation, and a high molecular weight complex I, probably consisting of a 1:1 enzyme:inhibitor stoichiometric complex, appeared on SDS-PAGE. After incubation for 30 minutes, complex II, which has been attributed to partially degraded complex,²⁵ was observed. When thrombin Tokushima and antithrombin III were incubated under identical conditions, only a small amount of complex I was formed after ten minutes. Following incubation for 30 minutes, the amount of complex I

increased, while no complex II was observed. These results suggest a decreased rate in the formation of the thrombin-antithrombin III complex, and little, if any, degradation of this complex with thrombin Tokushima, as compared to that of "α-thrombin." Figure 6B shows the time course of thrombin inhibition by antithrombin III in the absence of heparin. In both cases a time-dependent inhibition of the thrombin activity was observed. After ten minutes incubation the residual activity of "α-thrombin" reached a plateau with 45% of the initial activity, and thrombin Tokushima was inhibited by antithrombin III at a slower rate than that of "α-thrombin." Based on the residual thrombin activity after one minute incubation with antithrombin III, thrombin Tokushima was one third as effective as "α-thrombin" in forming the enzyme: inhibitor complex. A summary of these results is presented in Table 2.

DISCUSSION

Reports of congenital dysprothrombinemia have amounted to 16 families, which can be divided into three groups in terms of the localization of the functional defect within the molecule. The first group, typified by prothrombin San Juan,³ has an abnormality in the fragment 1-2 region of the molecule. The second group, which includes prothrombin Barcelona² and prothrombin Madrid,⁸ shows abnormalities in the sites cleaved by factor Xa. In the third group the thrombin portion of the molecule is abnormal, and examples of this group include prothrombin Quick,⁶ prothrombin Metz,⁹ and prothrombin Salakta.¹⁷

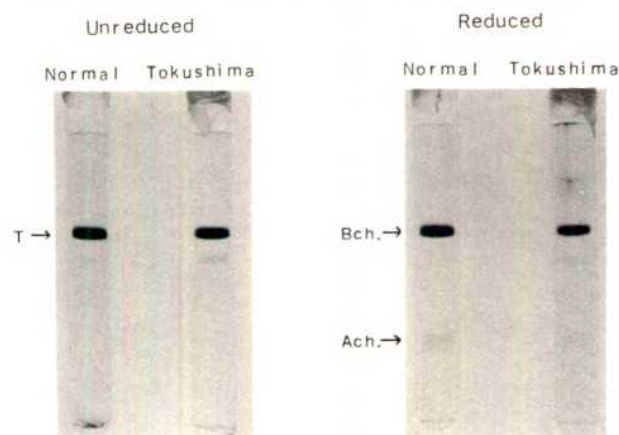


Fig. 4. Analysis of purified thrombins by SDS-PAGE. α-Thrombin, B chain, and A chain are abbreviated, respectively, as T, Bch, and Ach.

Table 1. Comparison of the Kinetic Parameters of "α-Thrombin" and Thrombin Tokushima

Titration with p-NPGB (%)	α-Thrombin 100	Thrombin Tokushima 95.7
Kinetic constants*		
K _m (μmol/L)	12.0	94.5
k _{cat} (sec ⁻¹)	93.5	49.4
k _{cat} /K _m (μmol/L ⁻¹ s ⁻¹)	7.79	0.52

*Kinetic constants were determined at 37 °C in 1 mL of 0.05 mol/L Tris-HCl (pH 8.0) containing 0.15 mol/L NaCl and 1 mmol/L CaCl₂.

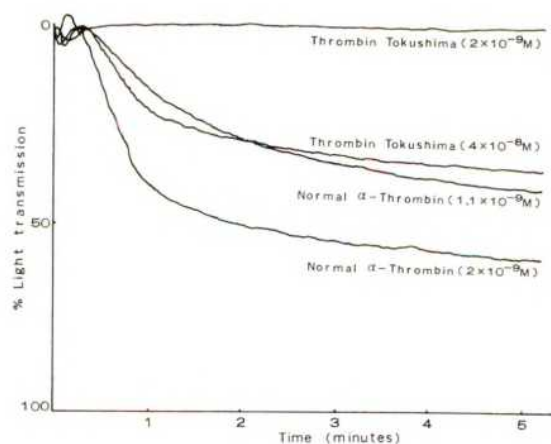


Fig 5. Thrombin-induced aggregation of gel-filtered platelets. Platelet concentration was 10^8 /mL in Tyrode's buffer. Thrombin Tokushima or " α -thrombin" was added at the final concentration indicated.

As we reported previously, prothrombin Tokushima is quantitatively adsorbed onto barium citrate, and the immunoelectrophoretic pattern of this prothrombin in plasma also appears to be normal.^{13,14} Moreover, the proteolytic processing of prothrombin Tokushima by factor Xa is indistinguishable from "prothrombin," as shown in this report. However, the prothrombin assay in plasma using the procoagulant fraction of *Echis carinatus* venom²⁶ and staphylocoagulase²⁷ as activating agents demonstrated only a small percentage of

Table 2. Summary of the Relative Activity (%) of Thrombin Tokushima with " α -Thrombin"

Fibrinogen	21.5
Factor XIII activation	69.9
Platelet aggregation	2.5
Antithrombin III inhibition	33.3

the normal clotting activity (data not shown). These observations suggest that the defect of prothrombin Tokushima may result from altered catalytic properties of the resulting thrombin. This prompted us to isolate thrombin produced upon activation of purified prothrombin Tokushima, and to characterize further its catalytic properties in a purified system.

Abnormal properties of thrombin Tokushima are observed in its kinetic parameter. The values of K_m and k_{cat} of thrombin Tokushima for Boc-Val-Pro-Arg-MCA are both abnormal, indicating that it differs from " α -thrombin" not only by its affinity for the substrate but also by its catalytic efficiency. The active-site titration value of thrombin Tokushima using *p*-NPGB is the same as that of " α -thrombin," suggesting that the residues that comprise the catalytic triad must be normal. These enzymatic properties are different from those of thrombin Quick⁶ and thrombin Metz.⁹ The active site titration of thrombin Metz using dansylarginine-*N*-(3-ethyl-1,5-pentanediol) amide (DAPA) has revealed that the fluorescence enhancement is only 34% of the maximum fluorescence observed with " α -thrombin," suggesting an abnormality of the catalytic site. Thrombin Quick has also been reported to have similar properties to those of thrombin Metz.

Thrombin Tokushima has no apparent platelet-aggregating activity at a concentration of 2×10^{-9} mol/L. However, at higher concentration of thrombin Tokushima (4×10^{-8} mol/L), incomplete aggregation of platelets was observed. In thrombin-mediated platelet activation, the catalytic site of thrombin and its binding site with platelets are both essential for this process. Concerning the thrombin-binding site in platelets, both high-affinity binding site ($K_d = 1.5 \times 10^{-9}$ mol/L) and low-affinity binding site ($K_d = 3 \times 10^{-7}$ mol/L) have been reported.²⁸ The decreased activity of thrombin Tokushima in initiating platelet aggregation in all likelihood reflects its reduced affinity to the high-affinity binding site in platelet, since titration of thrombin Tokushima with *p*-NPGB reveals a normal catalytic site.

Complex formation between antithrombin III and thrombin Tokushima also occurs at a slow rate in comparison to " α -thrombin." In addition, degradation of this complex is significantly less, indicating an abnormality of thrombin Tokushima in its interaction with antithrombin III.

In conclusion, the activities of thrombin Tokushima on synthetic tripeptide substrate, fibrinogen, factor XIII, platelets, and antithrombin III are reduced when compared with " α -thrombin," while active site titration with *p*-NPGB shows a normal value. Thus, in thrombin Tokushima the catalytic triad, consisting of the residues with His-43, Asp-99, and Ser-205 of the thrombin B chain,²⁹ seems to be structurally intact, but other residues in the vicinity of the catalytic site, eg, apolar binding sites,²⁹ may be structurally abnormal.

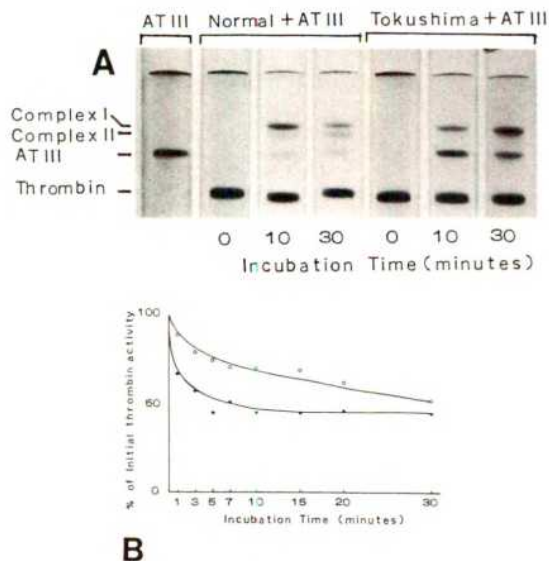


Fig 6. Inhibition of thrombin by antithrombin III, " α -thrombin" or thrombin Tokushima (1×10^{-6} mol/L) was incubated with antithrombin III (5×10^{-7}) in 0.05 mol/L Tris-HCl (pH 8.0) containing 0.1 mol/L NaCl. Serial aliquots (60 μ L) were removed for SDS-PAGE. (A) Antithrombin III (1.9 μ g), " α -thrombin" (2.2 μ g) and thrombin Tokushima (2.2 μ g) were applied to gels. (B) The remaining thrombin clotting activity was measured under the same conditions as described for Fig 1. The results are given in percentage of the initial thrombin activity v incubation time. Thrombin Tokushima (○) and " α -thrombin" (●).

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A Murine Antiglycoprotein Ib Complex Monoclonal Antibody, SZ 2, Inhibits Platelet Aggregation Induced by Both Ristocetin and Collagen

By Changgeng Ruan, Xiaoping Du, Xiaodong Xi, Peter A. Castaldi, and Michael C. Berndt

A new monoclonal antibody (MoAb), SZ 2, reactive with the human platelet glycoprotein Ib complex has been produced by the hybridoma technique. SZ 2 immunoprecipitated the components of the glycoprotein Ib complex, glycoprotein Ib and glycoprotein IX, from Triton-X-100-solubilized, periodate-labeled platelets. Western blot analysis indicated that the epitope for SZ 2 was on the α -subunit of glycoprotein Ib. Scatchard analysis of SZ 2 binding to formaldehyde-fixed, washed platelets revealed a single class of binding sites with $K_d = 6.6 \pm 3.3 \times 10^{-10}$ mol/L and $15,200 \pm 4,100$ binding sites per platelet (mean $\pm$ SD, $n = 10$). Intact antibody and its purified (Fab')₂ fragments not only inhibited the ristocetin-dependent binding of von Willebrand factor to platelets and ristocetin-induced platelet agglutination but also inhibited platelet aggregation induced by Type I collagen and platelet-activating factor (PAF). SZ 2 inhibited platelet serotonin and β -thromboglobulin release in response to these stimuli

and also platelet thromboxane A₂ formation in response to ristocetin and collagen. SZ 2 was without effect on platelet aggregation or release in response to other platelet stimuli such as ADP, thrombin, or arachidonic acid. The inhibition by SZ 2 of collagen- and PAF-induced platelet aggregation is surprising in that Bernard-Soulier syndrome platelets, which lack the glycoprotein Ib complex, respond normally to both these stimuli. SZ 2 was unreactive toward Bernard-Soulier syndrome platelets, as evaluated by fluorescence-associated cell sorting, and had no effect on the collagen- and PAF-induced aggregation of Bernard-Soulier syndrome platelets. The combined results suggest that the inhibition by SZ 2 of collagen- and PAF-induced aggregation of normal platelets is steric and are consistent with the glycoprotein Ib complex and the platelet collagen and PAF receptor(s) being adjacent in the human platelet plasma membrane.

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IN RESPONSE to vascular injury, the initial event in hemostasis involves the adhesion of platelets to collagenous and noncollagenous elements in the exposed subendothelium.^{1,2} At high shear flow, in capillaries and the microvasculature, both plasma von Willebrand factor and von Willebrand factor incorporated in the vessel wall subendothelial matrix play an important role in platelet adherence.^{3,4} At low shear rate in aorta and large blood vessels, platelet adhesion occurs independently of von Willebrand factor^{3,5} and presumably involves the direct adhesion of platelets to fibrils of collagen. There is now convincing evidence that the platelet membrane glycoprotein Ib complex is the receptor involved in the von Willebrand factor-dependent adhesion of platelets to exposed vascular subendothelium.⁶ Bernard-

Soulier syndrome platelets, which lack the components of the glycoprotein Ib complex,⁷ fail to bind von Willebrand factor,⁸ to agglutinate in the presence of ristocetin,^{9,10} and to adhere normally to subendothelium.⁵ The glycoprotein Ib complex is a heterodimer complex and contains both glycoprotein Ib and glycoprotein IX.¹¹ On SDS-polyacrylamide gels, glycoprotein Ib has an apparent molecular weight of 170,000 and consists of two disulfide-linked subunits, Ib_a and Ib_b, with apparent molecular weights of 135,000 and 25,000, respectively. Glycoprotein IX has an apparent molecular weight of 22,000 under both nonreducing and reducing conditions.¹¹ The binding domain for von Willebrand factor appears to be present on the α -subunit of glycoprotein Ib.^{6,12} The available evidence suggests that the glycoprotein Ib complex does not play an important role in platelet-collagen interaction. Bernard-Soulier syndrome platelets aggregate normally in response to collagen.¹³ Further, the murine monoclonal antibody (MoAb), AN 51, directed against the α -subunit of glycoprotein Ib,¹⁴ blocks ristocetin-induced, von Willebrand factor-dependent platelet agglutination but has no effect on platelet aggregation induced by types I or III collagen fibrils.¹⁵ In this study we describe a new murine MoAb, SZ 2, directed against the α -subunit of glycoprotein Ib, which not only inhibits ristocetin-induced, von Willebrand factor-dependent platelet agglutination but also collagen-induced and platelet-activating factor (PAF)-induced platelet aggregation. The combined data are consistent with the platelet glycoprotein Ib complex, the platelet collagen receptor, and the platelet PAF receptor being sterically adjacent in the human platelet plasma membrane.

MATERIALS AND METHODS

Materials. Bovine serum albumin (Fraction V), adenosine diphosphate, hypoxanthine, aminopterin, thymidine, A 23187, platelet-activating factor, and arachidonic acid were purchased from Sigma, St. Louis; sodium periodate (Univar) from Ajax Chemicals, Sydney, Australia; pristane from Aldrich, Milwaukee, WI; polyethylene glycol 4000 from Merck, Darmstadt, West Germany; ristocetin sulfate from Lundbeck, Copenhagen, Denmark; epinephrine

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from Wuxi No. 4 Pharmaceutical Plant, Wuxi, China; equine tendon collagen from Horm, Munich, West Germany; bovine von Willebrand factor from Speywood, England; bovine α -thrombin from Biochemical Products, Tianjing, China; RPMI 1640 medium from Gibco, Grand Island, NY; pepsin from Worthington, Freehold, NJ; protein-A-Sepharose CL-4B from Pharmacia, Sydney, Australia; Na ^{125} I from New England Nuclear, Boston; and ^{3} H-sodium borohydride from Amersham, Sydney, Australia. Platelet-activating factor (PAF), type I calf skin collagen, and human α -thrombin were the generous gifts of Dr Vargaftig, Paris; Dr Y. Legrand, Paris; and Dr J. W. Fenton, II, Albany, NY, respectively. Fluorescein-tagged goat antimouse IgG was from Cappel, Philadelphia; peroxidase-conjugated goat antimouse IgG was from Kirkegaard and Perry Laboratories, Gaithersburg, MD. Human von Willebrand factor was purified from commercial Factor VIII concentrate (CNTS, Paris, France) essentially as previously described¹⁶ and labeled by lactoperoxidase-catalysed radioiodination.

Platelet preparation and labeling. Platelet preparation from freshly drawn blood, platelet washing, and the periodate-labeling of platelets were each performed as previously described.¹⁷ For fluorescence-associated cell sorting and the analysis of SZ 2 binding to platelets, the platelets were washed and then fixed with 1% (wt/vol) paraformaldehyde essentially as previously described.¹⁵ Blood was also obtained from two patients, RS and LS, with the Bernard-Soulier syndrome.⁷ Platelets were isolated from these patients essentially as previously described.⁷

Monoclonal antibodies. The SZ 2 antibody (IgG₁) was prepared according to the method of Kochler and Milstein¹⁸ using 8-week-old BALB/C mice and washed platelets as the immunogen. Clones were assessed for antibody secretion into supernatant culture fluid by radioimmunoassay (RIA) using ^{125} I-labeled affinity-purified rabbit antimouse IgG and washed platelets essentially according to our previously described method.¹¹ Selected hybridomas were grown to confluence in 2-mL Costar plates and subsequently in 25-cm² plastic flasks. Cloning was performed by the limiting dilution method using 96-well microtitre plates with mouse peritoneal macrophage feeder layers. For SZ 2 this procedure was repeated a total of six times, prior to injection (10^7 cells) intraperitoneally (IP) into BALB/C mice previously primed with 0.5 mL of pristane. Resulting ascitic fluid was drained, centrifuged, and stored at -20°C .

IgG was purified from SZ 2 ascites fluid by chromatography on protein-A-Sepharose CL-4B as described by Ey et al.¹⁹ and was dialysed against either 0.01 mol/L sodium phosphate, 0.15 mol/L sodium chloride, pH 7.4 (PBS buffer) or 0.01 mol/L Tris, 0.15 mol/L sodium chloride, pH 7.4 prior to storage at -20°C . The purified IgG gave a single band at $\approx 150,000$ molecular weight as assessed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. SZ 2 (Fab')₂ fragments were prepared from SZ 2 IgG by pepsin digestion as described by Collier.²⁰ On SDS-polyacrylamide gel electrophoresis under nonreducing conditions, the SZ 2 (Fab')₂ gave a single band with a molecular weight of $\approx 100,000$. SZ 2 IgG was radioiodinated by the chloramine T method; $>95\%$ of the incorporated radioactivity was precipitable by 10% (wt/vol) trichloroacetic acid. Other MoAbs against the human platelet glycoprotein Ib complex have been previously reported: FMC 25 (IgG₁)^{11,14}; AN 51 (IgG₁)^{14,21}; WM 23 (IgG₁)¹¹; and AP 1.²² AP 1 was the generous gift of Dr T. J. Kunicki, Milwaukee. In some experiments SZ 21 (IgG₁) and WM 17 (IgG_{2b}) directed against the human platelet glycoprotein IIb/IIIa complex and FMC 18 (IgG₁) directed against an antigen on *Toxoplasma gondii*²³ were used as negative controls. IgG was purified from these MoAbs by chromatography on protein-A-Sepharose CL-4B essentially as described above.

Aggregation studies. The effect of MoAb IgG or (Fab')₂ fragments on platelet aggregation response was evaluated essentially as

previously described.¹⁴ Platelet aggregation was followed in PRP (3×10^8 platelets/mL, final concentration) stirred at 1,000 rpm at 37°C using either a Payton (Scarborough, Ontario) lumiaggregometer (model 1000) or a PAM-2 aggregometer (Danyang, China). Monoclonal antibody IgG (10 to 50 $\mu\text{g/mL}$, final concentration) or (Fab')₂ fragments (10 $\mu\text{g/mL}$) were added three minutes before the addition of stimuli at the following final concentrations: ADP, 2 $\mu\text{mol/L}$; epinephrine, 2.5 $\mu\text{mol/L}$; type I calf skin collagen, 10 to 20 $\mu\text{g/mL}$; equine tendon collagen, 0.5 to 1 $\mu\text{g/mL}$; ristocetin, 1.25 mg/mL; bovine vWF, 0.75 U/mL; A23187, 16 $\mu\text{mol/L}$; PAF, 0.3 $\mu\text{mol/L}$; and arachidonic acid, 200 $\mu\text{g/mL}$. Platelet aggregation in response to α -thrombin was performed using washed platelets suspended in Tyrode's solution (3×10^8 platelets/mL, final concentration); human α -thrombin, 0.05 to 0.1 U/mL; bovine α -thrombin, 0.125 U/mL). To evaluate platelet release the contents of the aggregometer cuvette were centrifuged five minutes after the addition of the platelet stimulus. Released serotonin was measured using the fluorescence method of Curzon et al.²⁴ β -Thromboglobulin was measured by RIA (the β -thromboglobulin assay kit was supplied by Shanghai 2nd Medical College, Shanghai, China). The formation of thromboxane B₂ was measured according to the procedure of Fitzpatrick,²⁵ using an iodinated tracer of thromboxane B₂. The anti-thromboxane B₂ antibody used for this assay was the generous gift of Dr J. Maclof (Unité 150 INSERM, Hôpital Lariboisière, Paris).

Inhibition of binding of von Willebrand factor to platelets. Binding of von Willebrand factor (vWF) to washed platelets was determined as previously described¹⁵ with minor modification. The binding assay for ^{125} I-labeled vWF to platelets was performed at room temperature in a final volume of 0.25 mL. Each tube contained 200 μL of washed platelets (2×10^8), 20 μL of PBS buffer or MoAb IgG (10 to 40 $\mu\text{g/mL}$, final concentration) in PBS buffer, 20 μL of ^{125} I-labeled vWF (25,000 cpm, 10 $\mu\text{g/mL}$ final concentration), and 10 μL of ristocetin (1 mg/mL, final concentration). After 30 minutes duplicate 100- μL aliquots of the reaction mixture were overlaid on 50 μL of dibutyl phthalate/dioctyl phthalate (2:1) in a narrow bore eppendorf tube and centrifuged at 12,000 g for three minutes. After careful aspiration of the supernatant, the pellet was counted for radioactivity in a gamma counter. Control experiments with this assay system confirmed that $>95\%$ of the binding of ^{125} I-labeled vWF to platelets was ristocetin-dependent.

Monoclonal antibody binding to platelets. Binding of SZ 2 IgG to formaldehyde-fixed, washed human platelets was assessed by equilibrium saturation analysis of ^{125} I-labeled SZ 2 IgG binding essentially as described by Powell-Jones et al.²⁶ Preliminary experiments established that equilibrium binding of SZ 2 IgG to platelets was achieved within 30 minutes under the assay conditions and remained at a constant level until 60 minutes. For the equilibrium saturation analysis of binding, 0.01 to 100 $\mu\text{g/mL}$, final concentration, of unlabeled SZ 2 IgG in PBS buffer was mixed with 10,000 cpm of ^{125} I-labeled SZ 2 in the same buffer and added to the platelet suspension (10^7 platelets/tube; in PBS buffer containing 0.3% (wt/vol) bovine serum albumin). The final volume was 200 μL /tube. After incubation at 37°C for 60 minutes, the platelets were separated by centrifugation, washed briefly in PBS buffer containing 0.1% (wt/vol) bovine serum albumin, and the pellet counted for radioactivity in a gamma counter. Control experiments confirmed that no dissociation of bound SZ 2 occurred within the time period of the platelet washing step. Nonspecific binding under the assay condition was $<2\%$. Binding data was analyzed according to the method of Scatchard.²⁷ The effect of a 100-fold excess of the antiglycoprotein Ib complex MoAbs, FMC 25, WM 23, AN 51, and AP 1, on the equilibrium binding of SZ 2 to platelets was performed using platelet-rich plasma prepared from venous blood collected into 3.2% sodium citrate as anticoagulant essentially as described by Collier et al.²⁰

Platelet immunofluorescence and examination by flow cytometry. Normal or Bernard-Soulier syndrome platelets (0.5 to 1.0×10^5) were incubated with saturating concentrations of SZ 2 IgG or WM 17 IgG at 20°C for ten minutes with intermittent agitation and then washed twice with Hanks' buffered saline solution (HBSS) containing 8 mmol/L potassium EDTA. As a control, first layer MoAb was substituted with an "irrelevant" MoAb, FMC 18, which reacts only with *T. gondii*. The washed platelets were then incubated with fluorescein-conjugated goat antimouse IgG (previously optimized for titre) at 20°C for ten minutes and then washed twice as described above. The amount of platelet-bound antibody was analyzed on a FACS 440 flow cytometer (Becton Dickinson, Mountain View, CA) using an argon ion laser at 488 nm and 200 mW and a $530 \pm 15\text{ nm}$ bandpass filter for log fluorescence determination. Data was stored and analyzed as LIST-mode files on a Becton Dickinson Consort 40 computer essentially as described by Adelman et al.²⁸

Immunoprecipitation. Immunoprecipitation using the mouse MoAb, SZ 2, employed the Triton X-100 lysate of periodate-labeled platelets and was performed as previously described.^{11,14} A mouse MoAb, WM 21, directed against human CALLA, was used as the negative control.

Electrophoresis. SDS-polyacrylamide gel electrophoresis, protein staining, fluorography, western blot analysis, and crossed immunoelectrophoresis were all performed as previously described.^{11,14}

RESULTS

Immune studies: Demonstration that SZ 2 is directed against the human platelet glycoprotein Ib complex. Immunization of mice with washed human platelets led to the establishment of a number of hybridoma cell lines secreting MoAbs to human platelets. One of these MoAbs, SZ 2, proved to be of special interest and was the subject of further detailed studies. The results of immunoprecipitation experiments using Triton-X-100-solubilized, periodate-labeled platelets are shown in Fig 1. SZ 2 immunoprecipitated the

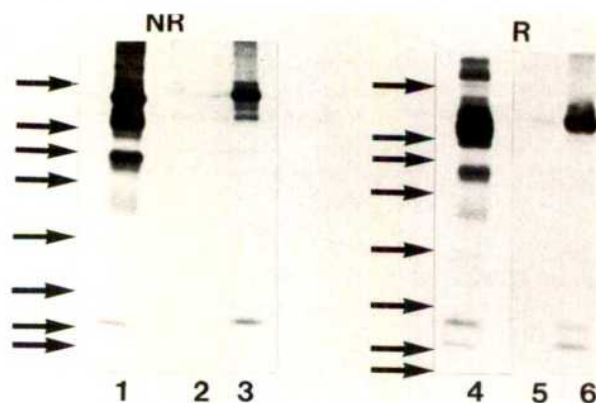


Fig 1. Fluorography of an exponential 5% to 20% SDS-polyacrylamide gel run under nonreduced (lanes 1 through 3) and reduced conditions (lanes 4 through 6) of the WM 21 and SZ 2 immunoprecipitates derived from the Triton X-100 lysate of periodate-labeled platelets; lanes 2 and 5, WM 21 immunoprecipitate; lanes 3 and 6, SZ 2 immunoprecipitate. Molecular weight markers in order of decreasing molecular weight are myosin ($200,000$), β -galactosidase ($130,000$), phosphorylase b ($94,000$), bovine serum albumin ($68,000$), ovalbumin ($43,000$), carbonic anhydrase ($29,000$), soybean trypsin inhibitor ($21,000$), and lysozyme ($14,000$).

components of the glycoprotein Ib complex (Fig 1 lane 3—nonreduced; lane 6—reduced). The glycoprotein assignments are based on the apparent molecular weights under nonreducing and reducing conditions¹¹ and their correspondence to the platelet glycoprotein profile in the platelet lysate: glycoprotein Ib, nonreduced mol wt = $170,000$, reduced mol wt = $135,000$ and $25,000$, most intensely labeled glycoprotein by the sialic-acid-specific periodate labeling procedure²⁹; glycoprotein IX, nonreduced and reduced mol wt = $22,000$. In addition, SZ 2 gave the same immunoprecipitation pattern when directly compared with other defined antiglycoprotein Ib complex MoAbs, FMC 25, WM 23, AN 51, and AP 1 (data not shown) and the same pattern of minor bands previously observed in immunoprecipitation experiments with FMC 25, AN 51, and quinine/quinidine drug-dependent antibodies.¹⁴ These comprise two minor bands of slightly lower apparent molecular weight than glycoprotein Ib in the nonreduced gel (lane 3) and a band of slightly higher molecular weight than glycoprotein Ib (lane 3) and glycoprotein Ib_a (lane 6) in the nonreduced and reduced gels, respectively. As previously discussed in detail,¹⁴ these bands probably derive from partial reduction of glycoprotein Ib and the co-precipitation of glycoprotein Ia with the glycoprotein Ib complex.

Since both the glycoprotein Ib complex and glycoprotein Ia co-immunoprecipitate with antiglycoprotein Ib complex MoAbs,^{14,20,30} presumably because of their mutual association with actin-binding protein,^{31,32} western blot analysis and crossed immunoelectrophoresis were performed to further characterize the reactivity of SZ 2. By western blot analysis, SZ 2 reacted with a single protein band of molecular weight $170,000$, nonreduced, and $135,000$, reduced (Fig 2), consis-

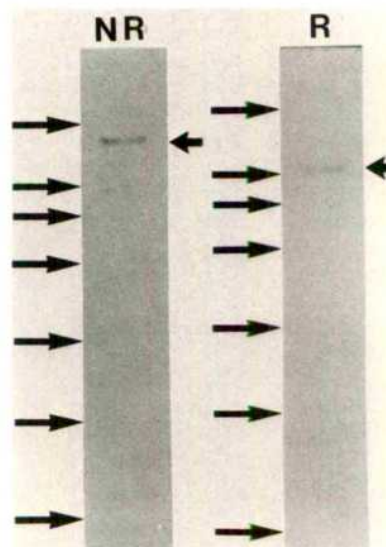


Fig 2. Western blot of SDS-polyacrylamide-gel-separated human platelet proteins probed with SZ 2. The experiment was performed as described under Materials and Methods. Molecular weight markers in order of decreasing molecular weight are myosin ($200,000$), β -galactosidase ($130,000$), phosphorylase b ($94,000$), bovine serum albumin ($68,000$), ovalbumin ($43,000$), carbonic anhydrase ($29,000$), and soybean trypsin inhibitor ($21,000$).

tent with SZ 2 recognizing an epitope on the α -chain of glycoprotein Ib. In contrast, glycoprotein Ia has an apparent molecular weight of 153,000 under nonreducing conditions and 167,000 under reducing conditions.³³ The reactivity of SZ 2 was further assessed by performing crossed immunoelectrophoresis with polyspecific rabbit antihuman platelet antisera and by incorporating radioiodinated SZ 2 IgG or WM 23 IgG in the intermediate gel in the second dimension. On autoradiography, SZ 2 gave an equivalent pattern to that observed with the antiglycoprotein Ib complex MoAb, WM 23.¹¹ There was no binding in the region of the gel previously shown to contain the immunoprecipitation arc for glycoprotein Ia³³ (data not shown).

Binding of ¹²⁵I-SZ 2 to fixed, washed platelets was rapid, being $\approx 50\%$ complete within ≈ 5 minutes and reached saturation within 30 minutes. Figure 3 shows the relationship between the amount of SZ 2 added and the amount bound. Binding of SZ 2 reached saturation with $\approx 0.3 \mu\text{g}$ of SZ 2 bound per 10^8 platelets. Scatchard analysis revealed a single class of binding sites (Fig 3) with $K_d = 6.6 \pm 3.3 \times 10^{-10}$ mol/L and $15,200 \pm 4,100$ binding sites per platelet (mean $\pm$ SD, $n = 10$). The results are comparable with the number of binding sites determined under identical conditions for the antiglycoprotein Ib complex MoAb, AN 51: $12,800 \pm 3,000$ (mean $\pm$ SD, $n = 10$)³⁴ and similar to the number of binding sites found for other antiglycoprotein Ib complex MoAbs with platelets in platelet-rich plasma.^{11,20} The binding of ¹²⁵I-SZ 2 ($1 \mu\text{g}/\text{mL}$) to platelets in platelet-rich plasma ($10^8/\text{mL}$) was inhibited by $>98\%$ by a 100-fold excess of unlabeled SZ 2, by $\approx 35\%$ by $100 \mu\text{g}/\text{mL}$ of AP 1, by $\approx 25\%$ by $100 \mu\text{g}/\text{mL}$ of AN 51, and by $<10\%$ by $100 \mu\text{g}/\text{mL}$ of WM 23 or FMC 25 (data not shown), indicating that SZ 2 bound to a unique epitope on the glycoprotein Ib complex relative to the other antiglycoprotein Ib complex MoAbs.

Effect of SZ 2 on platelet function. SZ 2 IgG (10 to $50 \mu\text{g}/\text{mL}$) had no effect on platelet aggregation induced by adenosine diphosphate, ADP ($2 \mu\text{mol}/\text{L}$), epinephrine ($2.5 \mu\text{mol}/\text{L}$), calcium ionophore, A23187 ($16 \mu\text{mol}/\text{L}$), arachidonic acid ($200 \mu\text{g}/\text{mL}$), human α -thrombin (0.05 to $0.1 \text{ U}/\text{mL}$), or bovine α -thrombin ($0.125 \text{ U}/\text{mL}$) (data not shown). In contrast, SZ 2 IgG ($10 \mu\text{g}/\text{mL}$) was a potent inhibitor of ristocetin-induced, von Willebrand factor-depen-

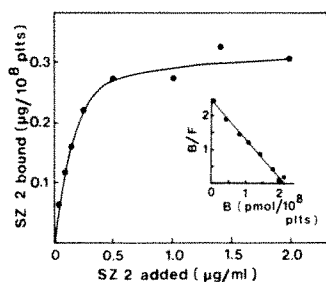


Fig 3. Effect of increasing concentration of ¹²⁵I-labeled SZ 2 on its steady-state binding to formaldehyde-fixed platelets. The insert shows a Scatchard plot of the same data with the $K_d \approx 4.4 \times 10^{-10}$ mol/L and the maximum number of sites $\approx 1.3 \times 10^4$ /platelet. The binding analysis was performed as described under Materials and Methods.

dent platelet agglutination (Fig 4A) and bovine von Willebrand factor-dependent platelet agglutination (Fig 4B). SZ 2 IgG directly inhibited ristocetin-induced platelet agglutination, since at saturating concentrations it blocked the ristocetin-dependent binding of human von Willebrand factor to washed human platelets by $\approx 80\%$. In this regard, it was less effective than AN 51 IgG, which completely blocked ristocetin-dependent von Willebrand factor binding under the same conditions (data not shown). SZ 21, directed against the human platelet glycoprotein IIb/IIIa complex, inhibited ristocetin-dependent von Willebrand factor binding by $<20\%$ (data not shown). Unexpectedly, SZ 2 IgG ($10 \mu\text{g}/\text{mL}$) was also a potent inhibitor of platelet aggregation induced by type I calf skin collagen ($20 \mu\text{g}/\text{mL}$) (Fig 4C) and by platelet-activating factor, PAF ($0.3 \mu\text{mol}/\text{L}$) (Fig 4D). SZ 2 IgG (10 to $50 \mu\text{g}/\text{mL}$) was also a strong inhibitor of platelet aggregation induced by the more potent collagen stimulus, equine tendon collagen (0.5 to $1.0 \mu\text{g}/\text{mL}$) (data not shown, cf Fig 6). Higher concentrations of equine tendon collagen (2 to $10 \mu\text{g}/\text{mL}$) partially overcame the inhibitory effect of SZ 2 IgG. However, the lag time before the onset of platelet shape change and aggregation was always increased in the presence of SZ 2 IgG, and the rate of aggregation was always slower than the rate in the absence of SZ 2. Since there is some evidence that the platelet Fc-receptor and the glycoprotein Ib complex may be sterically related in the human platelet membrane,³⁵ the aggregation experiments were repeated with SZ 2 (Fab')₂ fragments to eliminate the possibility that the Fc-region of the mouse immunoglobulin had a compounding effect on the observed results. The SZ 2 (Fab')₂ fragments, however, at $10 \mu\text{g}/\text{mL}$ gave similar inhibitory effects with respect to the intact immunoglobulin for platelet aggregation induced by ristocetin, by type I calf skin collagen, and by PAF (Fig 4). Early events in platelet activation in response to ristocetin, collagen, and PAF as defined by the platelet release reaction (Table 1) and thromboxane B₂ formation (Table 2) were also inhibited by SZ 2 IgG. In contrast, SZ 2 IgG had little or no effect on the release reaction or thromboxane B₂ formation in response to

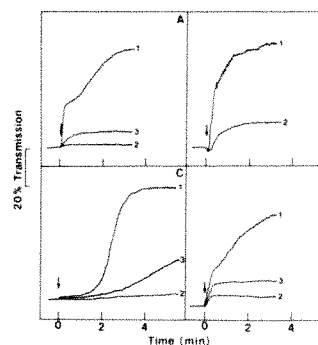


Fig 4. Effect of SZ 2 and its (Fab')₂ fragments on platelet aggregation. Trace 1, buffer control; trace 2, $10 \mu\text{g}/\text{mL}$ SZ 2; trace 3, $10 \mu\text{g}/\text{mL}$ SZ 2 (Fab')₂ fragments. Panel A, ristocetin ($1.25 \text{ mg}/\text{mL}$); panel B, bovine von Willebrand factor ($0.75 \text{ U}/\text{mL}$); panel C, type I calf skin collagen ($20 \mu\text{g}/\text{mL}$); panel D, platelet activating factor ($0.3 \mu\text{mol}/\text{L}$). The platelets were pre-incubated with buffer, SZ 2, or SZ 2 (Fab')₂ fragments for three minutes before the addition of the appropriate stimulus.

Table 1. Inhibition (%) of Platelet Function by SZ 2 IgG

Stimulus	Serotonin Release (% Inhibition)	β -Thromboglobulin Release (% Inhibition)
Ristocetin (1.25 mg/mL)	85	78
Collagen (20 μ g/mL)	72	47
PAF (0.3 μ mol/L)	73	78
Thrombin (1 U/mL)	< 10	< 10
Arachidonic Acid (200 μ g/mL)	< 10	< 10

Platelet-rich plasma or washed platelets were pre-incubated with SZ 2 IgG (10 μ g/mL), final concentration at 37 °C for three minutes before the addition of the appropriate stimulus. Measurement of serotonin and β -thromboglobulin release were as described under Materials and Methods. The results are the mean of three separate experiments.

α -thrombin or arachidonic acid. In addition, SZ 21, directed against the human platelet glycoprotein IIb/IIIa complex, had no significant effect on platelet thromboxane B_2 formation in response to either ristocetin or collagen (Table 2).

A series of experiments were also performed to contrast the effects of other antiglycoprotein Ib complex MoAbs (at 50 μ g/mL, final concentration) on platelet aggregation induced by ristocetin (1.5 mg/mL, final concentration), equine tendon collagen (1 μ g/mL, final concentration), PAF (0.1 μ mol/L), and ADP (5 μ mol/L). None of the antiglycoprotein Ib complex antibodies had any effect on ADP-induced platelet aggregation. Neither FMC 25 (epitope on glycoprotein IX)¹⁴ nor WM 23 (epitope on the α -chain of glycoprotein Ib)¹¹ inhibited platelet aggregation induced by either ristocetin or collagen. Consistent with the report of Ruan et al,¹⁵ AN 51 (epitope on the α -chain of glycoprotein Ib)¹⁴ completely inhibited ristocetin-induced, von Willebrand factor-dependent platelet agglutination but had no effect on collagen-induced platelet aggregation. Similar to SZ 2, AP 1 (epitope on the α -chain of glycoprotein Ib) also completely inhibited platelet aggregation induced by both ristocetin²² and collagen (data not shown). The inhibition of collagen-induced platelet aggregation by AP 1 was less pronounced at higher collagen concentrations in similar

Table 2. Effect of SZ 2 and SZ 21 on the Formation of Thromboxane B_2

Stimulus	Thromboxane B_2 (ng/3 $\times 10^6$ Platelets, $\pm$ SD)		
	Control	SZ 2 (10 μ g/mL)	SZ 21 (10 μ g/mL)
Arachidonic Acid (330 μ mol/L)	1210 $\pm$ 640	1060 $\pm$ 380	1610 $\pm$ 420
Thrombin (0.125 U/mL)	151 $\pm$ 68	169 $\pm$ 72	203 $\pm$ 95
Collagen (25 μ g/mL)	42 $\pm$ 15	9 $\pm$ 3*	30 $\pm$ 19
Ristocetin (1.25 mg/mL)	27 $\pm$ 13	9 $\pm$ 3*	22 $\pm$ 9

Platelet-rich plasma or washed platelets were pre-incubated with SZ 2 IgG or SZ 21 IgG at 37 °C for three minutes before the addition of the appropriate stimulus. Measurement of thromboxane B_2 formation was as described under Materials and Methods. The results are the mean of six separate experiments.

* $p < 0.01$ for SZ 2 IgG compared to buffer control or SZ 21 IgG by Student's t test.

manner to that observed with SZ 2. PAF-induced platelet aggregation was not inhibited by AN 51, WM 23, FMC 25, AP 1, or FMC 18 (directed against *T gondii*).

Effect of SZ 2 on Bernard-Soulier syndrome platelets. The inhibition by SZ 2 of collagen-induced aggregation is inexplicable in the sense that Bernard-Soulier syndrome platelets, which lack the components of the glycoprotein Ib complex,⁷ aggregate normally in response to collagen.¹³ There are two likely possibilities consistent with these results. One possibility is that SZ 2 is directed against an epitope present on more than one platelet surface membrane protein: SZ 2 could be directed against both the α -chain of glycoprotein Ib and a platelet collagen receptor. Alternatively, the inhibition of collagen-induced aggregation by SZ 2 could be steric with the binding of SZ 2 to the glycoprotein Ib complex affecting the normal interaction of collagen with a proximal collagen receptor. The first possibility seems unlikely since immunoprecipitation, western blot analysis, and crossed immunoelectrophoresis strongly suggest that SZ 2 is directed against an epitope present only on the glycoprotein Ib complex. Furthermore, another antiglycoprotein Ib complex antibody, AP 1, directed against a distinct epitope to SZ 2 on the glycoprotein Ib complex, was also found to inhibit collagen-induced platelet aggregation. To help further distinguish between these two possibilities we examined the reactivity of SZ 2 toward Bernard-Soulier syndrome platelets. The reactivity of SZ 2 toward normal and Bernard-Soulier syndrome platelets as evaluated by fluorescence-associated cell sorting is shown in Fig 5. The upper panels show the reactivity of normal and Bernard-Soulier syndrome platelets toward FMC 18, directed against *T gondii* and establish the negative controls for background fluorescence by this method. The middle panels show the reactivity of normal and Bernard-Soulier syndrome platelets toward WM 17, directed against the human platelet glycoprotein IIb/IIIa complex and establish the controls for positive platelet

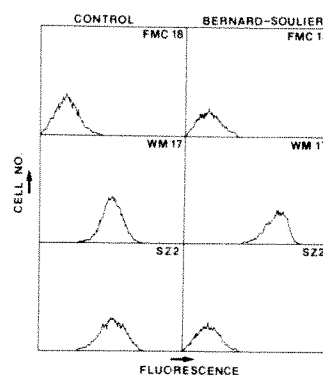


Fig 5. Immunofluorescence profiles of normal (control) and Bernard-Soulier syndrome platelets (BSS) stained with FMC 18 (against *Toxoplasma gondii*), WM 17 (against glycoprotein IIb/IIIa), and SZ 2 (against glycoprotein Ib complex). Formaldehyde-fixed platelets incubated with the respective MoAb and fluorescein-labeled second antibody were analyzed by fluorescence flow cytometry. Each single parameter histogram, cell number ν log fluorescence (arbitrary units), was derived from the analysis of 10,000 platelets. The experimental procedure and data analysis were performed as described under "Materials and Methods."

immunofluorescence. Although SZ 2 was positive against normal platelets, the immunofluorescence seen with Bernard-Soulier syndrome platelets was indistinguishable from that observed with the negative control, confirming that SZ 2 was solely directed against an epitope present on the glycoprotein Ib complex. Furthermore, although SZ 2 inhibited collagen-induced platelet aggregation with control platelets, it was without effect on the collagen-induced aggregation of Bernard-Soulier syndrome platelets at any collagen concentration (Fig 6). The latter result strongly suggests that SZ 2 does not bind directly to a platelet-collagen receptor and supports the concept that the human platelet glycoprotein Ib complex and the platelet-collagen receptor are proximal in the human platelet membrane. Similar results were obtained with PAF-induced platelet aggregation. Although SZ 2 (50 $\mu\text{g}/\text{mL}$) was a potent inhibitor of the aggregation of normal platelets by PAF (40 nmol/L), SZ 2 had no effect on the PAF-induced aggregation of Bernard-Soulier syndrome platelets at any PAF concentration (data not shown).

DISCUSSION

In response to vascular injury, platelets rapidly adhere to the exposed vascular subendothelium.¹ At high shear flow, one adhesion mechanism appears to involve von Willebrand factor^{3,4} and a specific von Willebrand factor receptor on the human platelet membrane surface, the glycoprotein Ib complex.² For example, Bernard-Soulier syndrome platelets, which apparently genetically lack the components of the glycoprotein Ib complex, glycoprotein Ib and glycoprotein IX,⁷ fail to bind von Willebrand factor,⁸ to agglutinate in the presence of ristocetin,^{9,10} and to adhere normally to subendothelium at high shear flow.⁵ In recent years a number of antiglycoprotein Ib murine MoAbs have been described (AN 51,¹⁵ 6D1,²⁰ and AP 1²²) that block the ristocetin-dependent binding of von Willebrand factor to human platelets. The inhibition of von Willebrand factor binding to platelets correlates well with the failure of MoAb-treated platelets to agglutinate with ristocetin²⁰ or to adhere normally to exposed vascular subendothelium at high shear flow.¹⁵ In this study we have described a new murine MoAb, SZ 2, directed against an epitope on the α -subunit of glycoprotein Ib (Fig

2). Like the previously reported murine MoAbs, AN 51, 6D1, and AP 1, SZ 2 and its (Fab')₂ fragments were potent inhibitors of both ristocetin-induced, human von Willebrand factor-dependent and bovine von Willebrand factor-dependent platelet agglutination (Fig 4) and inhibited the ristocetin-dependent binding of human von Willebrand factor to platelets by $\approx 80\%$. SZ 2 had no effect on platelet aggregation induced by ADP, epinephrine, A23187, arachidonic acid, and by human or bovine α -thrombin. Unexpectedly, however, SZ 2 and its (Fab')₂ fragments were potent inhibitors of platelet aggregation induced by collagen and by PAF (Fig 4). SZ 2 appeared to interfere with an early event in collagen-platelet interaction. SZ 2 inhibited the platelet release reaction in response to collagen as measured by the extent of secretion of dense body serotonin or α -granule β -thromboglobulin (Table 1) and also inhibited the mobilization of arachidonic acid from platelet membrane phospholipid as measured by thromboxane B₂ formation (Table 2). With high concentrations of equine tendon collagen (2 to 10 $\mu\text{g}/\text{mL}$), SZ 2 consistently prolonged the lag time between the addition of the collagen fibrils and the onset of platelet shape change. Although there is some evidence that collagen-induced platelet aggregation may be partially von Willebrand factor-dependent at threshold concentrations of collagen,^{36,37} the inhibition of the binding of von Willebrand factor to platelets by SZ 2 is unlikely to represent the mechanism by which SZ 2 inhibits collagen-dependent platelet aggregation. AN 51, which completely blocks the binding of von Willebrand factor to platelets, has no effect on collagen-induced platelet aggregation (this study).¹⁵ Similarly, 3F8, a MoAb against human von Willebrand factor, completely blocks ristocetin-induced, human von Willebrand factor-dependent platelet agglutination¹⁶ but has no effect on collagen-induced platelet aggregation at collagen concentrations at which SZ 2 completely inhibits platelet aggregation (Berndt and Gregory, unpublished observations).

The inhibition of collagen-induced platelet aggregation by SZ 2 is surprising in the sense that Bernard-Soulier syndrome platelets, which lack the glycoprotein Ib complex, aggregate normally in response to collagen (this study, Fig 6).¹³ One possible explanation for this apparent discrepancy is that SZ 2 is directed against a binding site on more than one platelet membrane surface protein, ie, SZ 2 could be directed against an epitope present on a platelet collagen receptor as well as the platelet membrane glycoprotein Ib complex. The available evidence, however, is inconsistent, with this being the probable explanation for the observed results since detailed immunochemical analysis indicated that SZ 2 only recognized the glycoprotein Ib complex and since SZ 2 did not inhibit the collagen-induced aggregation of Bernard-Soulier syndrome platelets (Fig 6). An alternate possibility to explain the inhibition of collagen-induced aggregation by SZ 2 is that the glycoprotein Ib complex and the platelet collagen receptor are proximal in the platelet membrane, ie, the binding of SZ 2 or its (Fab')₂ fragments to the α -subunit of glycoprotein Ib sterically interferes with the normal interaction of collagen with an adjacent collagen receptor or membrane protein required for normal collagen-dependent platelet activation. There are three lines of evi-

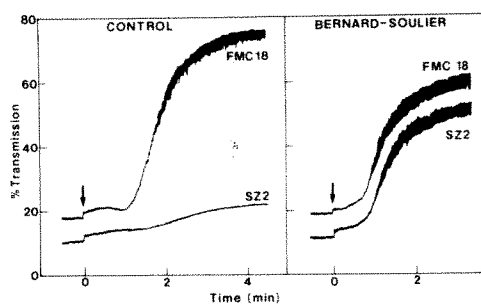


Fig 6. Effect of FMC 18 and SZ 2 on the collagen-induced aggregation of control and Bernard-Soulier syndrome platelets. Control (3×10^8 platelets/mL, final concentration) and Bernard-Soulier syndrome platelets (1.5×10^8 platelets/mL) were pre-incubated with 50 $\mu\text{g}/\text{mL}$ of either FMC 18 IgG or SZ 2 IgG for three minutes prior to the addition of 1 $\mu\text{g}/\text{mL}$ (control) or 0.5 $\mu\text{g}/\text{mL}$ (Bernard-Soulier) of equine tendon collagen.

dence consistent with this possibility. First, consistent with the preliminary observations of Pidard et al,³⁸ AP 1, directed against a distinct epitope on the glycoprotein Ib complex from that of SZ 2 (since AP 1 and SZ 2 only partially crossblock), also inhibited collagen-induced platelet aggregation. Second, SZ 2 only inhibited collagen-induced platelet aggregation when bound to glycoprotein Ib, since SZ 2 had no effect on the collagen-induced aggregation of Bernard-Soulier syndrome platelets. A third line of evidence derives from the recent data of Nieuwenhuis et al³⁹ and of Fox.^{31,32} Nieuwenhuis et al have described a patient whose platelets failed to react with collagen and lacked a specific membrane protein, glycoprotein Ia. Fox has recently shown that in unstimulated platelets at least one subunit of the glycoprotein Ib complex spans the platelet membrane and is bound to a platelet membrane endoskeleton via actin-binding protein.^{31,32} Glycoprotein Ia also apparently spans the platelet membrane and, like glycoprotein Ib, is also bound to the platelet endoskeleton via actin-binding protein.^{31,32} These results suggest that glycoprotein Ia and the glycoprotein Ib complex may be normally associated in the human platelet membrane because of their mutual interaction with actin-binding protein and provide an explanation for the inhibition by SZ 2 of both ristocetin-induced, human von Willebrand factor-dependent platelet agglutination and collagen-induced platelet aggregation. Similar arguments apply for the inhibition of PAF-induced platelet aggregation by SZ 2. SZ 2 was found to be a potent inhibitor of both the PAF-induced platelet release reaction (Table 1) and PAF-induced platelet aggregation (Fig 4). However, Bernard-

Soulier syndrome platelets aggregated normally in response to platelet activating factor, and this aggregation was unaffected by SZ 2. This suggests that, similar to the glycoprotein Ib complex and the platelet collagen receptor, the glycoprotein Ib complex and the platelet PAF receptor (or a membrane protein required for normal PAF-dependent platelet activation) may also be proximal in the plane of the platelet membrane.

The combined evidence suggests that the antiglycoprotein Ib complex MoAb, SZ 2, inhibits platelet aggregation induced by both ristocetin and collagen because the platelet glycoprotein Ib complex and the platelet collagen receptor may be sterically adjacent in the human platelet plasma membrane. Houdijk et al have recently demonstrated that the adhesion of platelets to collagen substrata at high shear flow is von Willebrand factor-dependent.⁴⁰ It is interesting to speculate that the possible steric relationship between the platelet von Willebrand receptor and the platelet collagen receptor suggested by the current study may be relevant to these observations. Future studies should help resolve this question and help delineate the precise molecular role of glycoprotein Ia in collagen-induced platelet aggregation.

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Human Erythroleukemia Cells Adhere to Fibronectin: Evidence for a M_r 190,000-Receptor Protein

By Ismo Virtanen, Jari Ylänné, and Tapio Vartio

Human erythroleukemia cells (K562) adhered rapidly on fibronectin (Fn)-coated growth substratum under serum-free conditions. The adhesion could be quantitatively inhibited by the synthetic peptide Arg-Gly-Asp-Ser (RGDS) and upon hemin-induced differentiation or trypsinization of the cells. Many of the cells also displayed rapid spreading that led to a redistribution of F-actin into spreading edges and in many cells also to a formation of typical actin fibers attaching to the ventral aspect of the cells. The spreading of the cells was inhibited by cytochalasin B but not by microtubule-disrupting drugs, suggesting an active role for the microfilament system in the spreading process. Direct overlay assay of electrophoretically separated polypep-

tides with ¹²⁵I-Fn showed that in K562 cells there is a major M_r 190,000 Fn-binding protein that is lost upon differentiation. A similar overlay assay with purified plasma and cellular Fns followed by immunostaining with anti-Fn antibodies revealed a reaction with a similar polypeptide. The binding of Fns on the nitrocellulose sheets could be inhibited and the bound Fn eluted by using the RGDS peptide. From octylglucoside extracts of radioactively surface-labeled cells, distinct M_r 190,000/185,000 membrane glycoproteins bound to Fn-heptapeptide-Sepharose, further suggesting that the M_r 190,000 polypeptide would be the Fn-receptor of the K562 cells.

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MUCH INTEREST has been devoted to the mechanisms of fibronectin(Fn)-mediated adhesion of cells to culture substratum.¹ Results from several laboratories indicate that this process takes place via receptor-like proteins present in the surface membrane of diverse cells, including fibroblasts,²⁻⁶ macrophages,⁷ and platelets.⁸ In fibroblasts the receptor appears to be an M_r 140,000 polypeptide,^{2,6} whereas a distinct M_r 100,000 has been characterized in macrophages⁷ and an M_r 110,000 in platelets.⁸

Murine erythroleukemia (MEL) cells⁹⁻¹¹ and reticulocytes¹² also appear to exhibit an adhesion capacity to Fn-coated substrata and to cells that have Fn-matrix. This capacity appears to be lost upon dimethyl sulfoxide (DMSO)-induced differentiation of the MEL cells¹¹ as well as upon further maturation of the red blood cells.¹² Recently Patel and Lodish¹¹ suggested that an M_r 140,000 protein would be responsible for the Fn-binding of MEL cells and would thus be a candidate for "erythroid Fn-receptor." These results suggested to Patel and Lodish^{10,11} and Patel et al.¹² that Fn-mediated adhesion might play a role in the maturation of precursors of red blood cells. The results of the present study show that human K562 erythroleukemia cells both adhere and spread on exogenous Fn and express a distinct M_r 190,000 Fn-binding protein that may mediate this process.

MATERIALS AND METHODS

Cell lines and adhesion experiments. Human erythroleukemia K562¹³⁻¹⁵ cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) and antibiotics.

Fibronectin (pFn) was purified from human plasma as described by Engvall and Ruoslahti.¹⁶ Multiwell dishes and Petri dishes (Nunc, Copenhagen, Denmark) were coated by pFn (20 µg mL⁻¹) for two hours. Then the dishes were washed with NaCl-P buffer (140 µmol/L NaCl, 10 µmol/L sodium phosphate, pH 7.4). Laminin from EHS sarcoma, used to coat culture dishes as above, was from Bethesda Research Laboratories (Gaithersburg, MD). Similar amounts of cells were plated in all experiments in serum-free RPMI medium. In some experiments the cells were induced to differentiate by exposing them to hemin (20 µmol/L; Sigma, St. Louis).¹⁷ The induced state was monitored by using both the benzidine staining for hemoglobin and monoclonal antibodies (MoAbs) binding to erythroid α- and β-spectrins (HE53DA7; Virtanen et al, in preparation) in immunoblotting (see below). Poly-L-lysine (20 µg mL⁻¹; Sigma) was used to adhere K562 cells nonspecifically in some experiments by preincubation of the coverslips one hour before plating of the cells. In some experiments the cells were exposed to trypsin (0.25%; Sigma) for 15 minutes before plating, to cytochalasin B (1 µg mL⁻¹; Sigma) or to demecolcine (1 µg mL⁻¹; Sigma) for one to 24 hours. The synthetic peptide Arg-Gly-Asp-Ser (RGDS, Peninsula Laboratories, Belmont, CA) was used for cell adhesion experiments at a concentration of 0.1 to 0.5 mg mL⁻¹ in serum-free RPMI medium.

SDS-PAGE and immunoblotting. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed under reducing conditions according to Laemmli¹⁸ using 6.5% slab gels. Polypeptides were transferred onto nitrocellulose sheets according to Towbin et al.¹⁹ After the transfer the sheets were incubated overnight in a 3% bovine serum albumin-NaCl-P buffer and processed for overlay experiments (see below) or immunostaining. For immunostaining of the nitrocellulose sheets the following antibodies were used: monoclonal spectrin (HE53DA7) and Fn (against the cell-binding site; N295, Mallinkrodt, St. Louis) as well as polyclonal Fn antibodies. The sheets were exposed to the antibodies for 60 minutes, washed, and were then reacted with peroxidase-coupled rabbit antimouse or sheep antirabbit IgG antisera (Dakopatts, Glostrup, Denmark), respectively. After washing, the peroxidase reaction was developed as described by Towbin et al.¹⁹ The molecular weights were estimated by using the low molecular weight calibration kit (Pharmacia, Uppsala, Sweden) and in some experiments by using reduced pFn (M_r 220 000) and myosin (M_r 200 000) from bovine Purkinje fibers as additional standards.

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Fn overlay and radioactive surface labeling experiments. For overlay experiments, fibronectin from human fibroblasts (cFn, Bethesda Research Laboratories, Gaithersburg, MD) was iodinated (^{125}I) by using the technique of Bolton and Hunter²¹ as modified.²² The reagent was from Radiochemical Centre, Amersham, UK. The overlay assay of nitrocellulose sheets was performed as described earlier for ^{125}I -cFn²³ and ^{125}I -calmodulin.²⁴

Alternatively, the nitrocellulose sheets were first exposed to purified pFn or cFn ($20\text{ }\mu\text{g mL}^{-1}$) for 60 minutes, washed, and were then exposed to monoclonal or polyclonal antibodies to Fn. After washing, the bound antibodies were detected by peroxidase immunostaining as above. In a set of experiments the RGDS synthetic peptide was applied on the sheets together with Fns at a concentration of 0.5 mg mL^{-1} to inhibit the binding of the proteins. The peptide was used also at the same concentration to elute bound Fns from the nitrocellulose sheets. For this purpose the peptide was applied on the sheet for 30 minutes.

For radioactive surface labeling, the neuraminidase-galactose oxidase-sodium borohydride technique was used.²⁵ After electrophoresis the gels were processed for fluorography.²⁶

Affinity-binding of cell surface glycoproteins to Fn-synthetic peptide-Sepharose. The synthetic heptapeptide, Gly-Arg-Gly-Asp-Ser-Pro-Cys (GRGDSPC), which corresponds to the cell-adhesion promoting region of Fn and which can autoinhibit Fn-function,²⁷ was purchased from Peninsula Laboratories. A portion (0.25 mg) of the peptide was coupled to 1.0 mL of wet, activated thiol-Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. For affinity binding experiments, surface-labeled K562 cells were extracted in 200 mmol/L octylglucoside (Sigma) in NaCl-P buffer, supplemented with 3 mmol/L phenylmethyl sulfonyl fluoride at 0°C for 30 minutes. Insoluble material was removed by centrifugation at $1400 \times g$ for 15 minutes. Aliquots of the extract were then applied on peptide-thiol-Sepharose for two hours in the presence or absence of the synthetic tetrapeptide RGDS (0.5 mg mL^{-1}).²⁸ Thereafter the Sepharose-beads were washed with the octylglucoside-buffer and the bound proteins were eluted with the electrophoresis sample buffer for analysis in SDS-PAGE.

Fluorescence microscopy. For fluorescence microscopy the cells on culture dishes or glass coverslips were fixed in 3.5% paraformaldehyde for five minutes. Then they were permeabilized in 0.01% NP40 and exposed to NBD-phalloidin (Molecular Probes, Junction City, OR), a fluorescent probe for F-actin.³⁰ In some experiments the cells were fixed in methanol, cooled to -20°C , and were then first exposed to mouse vinculin antiserum, raised against vinculin purified from chicken gizzards as described.³¹ Then the specimens were exposed to fluorescein isothiocyanate-coupled rabbit antimouse IgG antiserum (Cappel Laboratories, Cochranville, PA). A Zeiss Universal microscope, equipped with epiilluminator IIIRS, was used to examine the specimens.

RESULTS

When exponentially growing K562 cells were plated in serum-free medium on tissue culture wells, precoated with pFn, over 95% of the cells attached firmly within ten minutes (Fig 1A) and could not be detached even by vigorous shaking. Within one to two hours, over 30% of the adhered K562 cells displayed distinct flattening and spreading (Fig 1B). Pre-exposure of the cells to trypsin or plating in the presence of the synthetic peptide RGDS, the minimum cell-binding sequence of Fn,²⁶⁻²⁸ inhibited quantitatively the attachment of the cells on pFn-coated dishes. Furthermore, exposure of the cells to cytochalasin B completely prevented

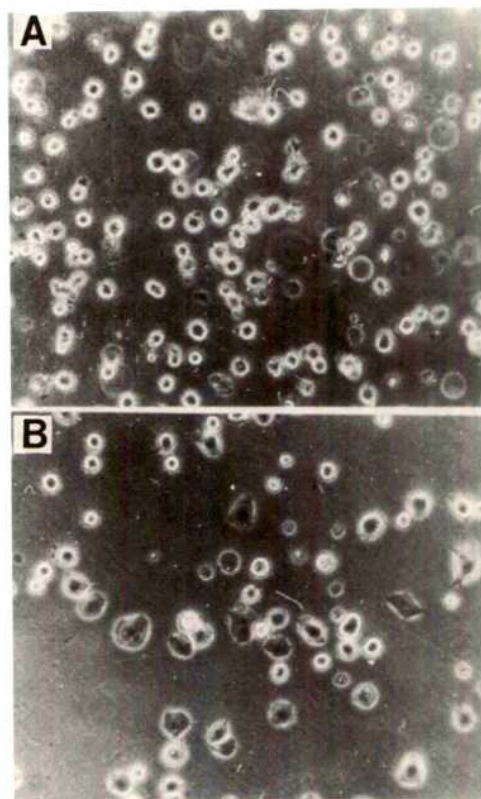


Fig 1. Phase-contrast microscopy on adhesion and spreading of the K562 cells on Fn-coated growth substratum. Within ten minutes after plating, most of the cells were adhered (A), and over 30% of them showed distinct spreading within one to two hours (B). Original magnification $\times 200$.

the spreading but not the adhesion of the cells, whereas exposure to demecolcine did not have any apparent effect on these processes. In control experiments, only occasional cells bound to plain plastic substratum or on laminin-coated dishes, and all the cells could be brought into suspension by gentle agitation. Results from adhesion experiments on K562 cells under different culture conditions are summarized in Table 1.

In rounded K562 cells, labeling with NBD-phalloidin revealed an even rim-like surface staining (Fig 2A). In cells spreading on pFn, NBD-phalloidin gave a reaction with microspike- and lamellae-like structures at the spreading edges of the cells (Fig 2B). In the spread cells, distinct NBD-phalloidin-positive cytoplasmic fibrils were seen (Fig 2C), and antibodies to vinculin revealed related ventral membrane-associated plaques in the adhering cells (Fig 2D).

When K562 cells were induced to differentiate with hemin,¹⁷ they lost the ability to attach on pFn-coated substratum and remained floating in the medium. In immunoblotting of the electrophoretically separated polypeptides, the induced but not the control K562 cells showed a distinct doublet of spectrin polypeptides, as revealed with the MoAb HE53DA7 (Fig 3, lanes 5, 6).

We then studied putative receptor proteins for Fn in K562

Table 1. Adhesion and Spreading of K562 Cells on Different Substrata

Growth Substratum and Culture Condition	Cells Adhered (10 min)	Cells Spread (2 h)
20 $\mu\text{g mL}^{-1}$ pFn	95%	30%–35%
20 $\mu\text{g mL}^{-1}$ laminin	<5%*	0
plastics	<5%*	0
pFn + trypsinized cells	<5%*	0
pFn + RGDS	<5%*	0
pFn + hemin-induced cells	<5%*	0
pFn + cytochalasin B	95%	0
pFn + demecolcine	95%	30%–35%

Culture dishes were coated with the reagents indicated and the cells treated as described in the "Materials and Methods" section. The number of adhered or spread cells were calculated by visual inspection.

*Adhered cells could be brought into suspension by a gentle agitation.

cells by using direct overlay assays in which the polypeptides of the cells were first separated in SDS-PAGE and then transferred onto nitrocellulose sheets. Then the sheets were either exposed to ^{125}I -cFn and the bound protein was detected by autoradiography or the lanes were exposed to pFn or cFn and the bound proteins were detected by immunostaining. Using ^{125}I -cFn, a distinct ca M_r 190,000 polypeptide could be revealed in K562 cells (Fig 3, lane 2), whereas no binding proteins were detected in hemin-differentiated K562 cells (Fig 3, lane 4), which expressed erythroid α - and β -spectrins, as revealed by immunoblotting with a MoAb (Fig 3, lane 6). When unlabeled pFn or cFn were applied on the nitrocellulose sheets and their binding was detected, a prominent M_r 190,000 polypeptide could again be revealed in K562 cells with both monoclonal (Fig 4, lane 2, pFn; lane 3, cFn) and polyclonal Fn antibodies (Fig 4, lane 4, pFn). When the RGDS synthetic peptide was included in the overlay experi-

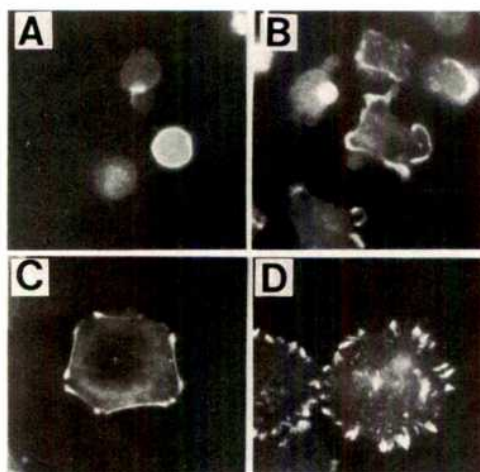


Fig 2. Localization of F-actin in control K562 cells adhered on poly-L-lysine-coated substratum (A), spreading cells 30 minutes after plating on pFn-coated dishes (B), and spread cells two hours after plating (C). Note the even cell surface-associated staining in (A), more bright staining of the spreading edges in (B), and the reaction with stress fibre-type of filaments in (C). In the spread cells, antibodies to vinculin visualize typical ventral plaques (D). Original magnification $\times 600$.

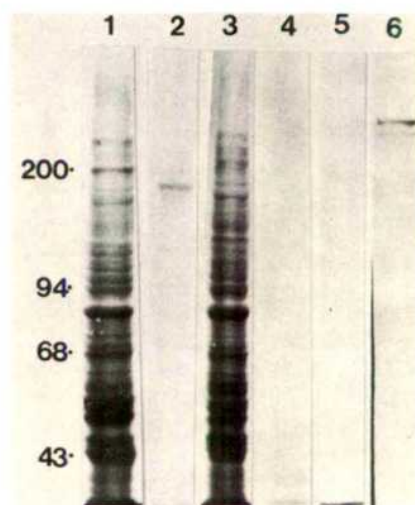


Fig 3. SDS-PAGE separated polypeptides of control K562 cells (lanes 1, 2, 5) and hemin-induced K562 cells (lanes 3, 4, 6) transferred onto nitrocellulose sheets and stained for protein, ^{1,3} exposed to ^{125}I -cFn (lanes 2, 4) or immunostained for α - and β -spectrins (lanes 5, 6). Note the distinct Fn-binding polypeptide in lane 2, lack of the reaction in lane 4, and two polypeptides at M_r 240,000 and 220,000 in lane 6. Molecular weights of the control proteins are indicated on the left.

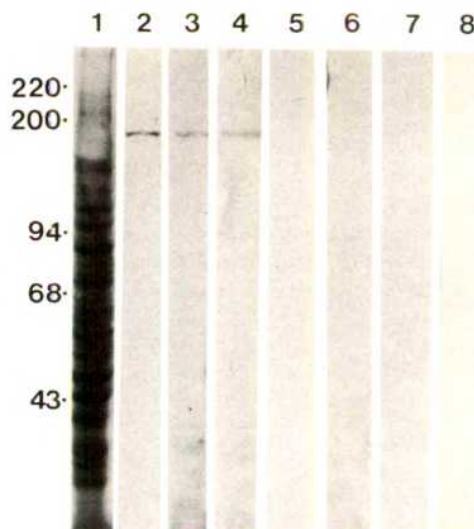


Fig 4. SDS-PAGE separated polypeptides of K562 cells transferred onto nitrocellulose sheets and exposed to unlabeled Fns (lanes 2 to 6) or immunostained for Fn (lanes 7, 8). Lane 1 shows the corresponding amido black staining for proteins. When exposed to either pFn (lane 2) or cFn (lane 3), immunostaining with monoclonal Fn antibodies revealed a reaction with a distinct M_r 190,000 polypeptide, and immunostaining with polyclonal Fn antibodies revealed a similar polypeptide (lane 4, pFn). No reaction was obtained when the RGDS peptide was incubated together with Fns (lane 5, pFn) or was applied on sheets pre-exposed to Fns (lane 6, pFn). Control immunostaining experiments revealed that neither the monoclonal (lane 7) nor the polyclonal (lane 8) Fn antibodies reacted with any polypeptides of K562 cells. Molecular weights of the control proteins are indicated on the left.

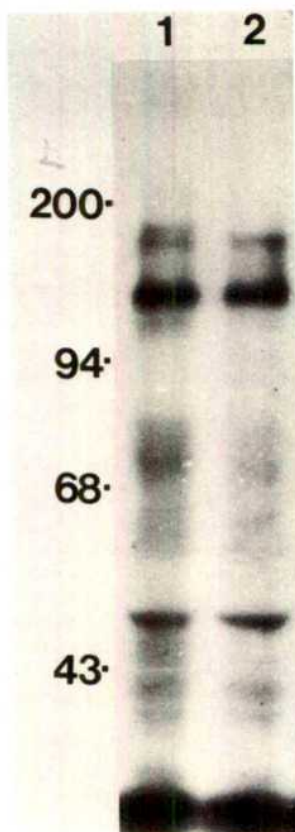


Fig 5. Autoradiogram of SDS-PAGE-separated polypeptides of the surface-labeled control (lane 1) and trypsinized K562 cells (lane 2). Note the M_r 190,000/185,000, 120,000, 80,000/70,000, and 50,000 polypeptides of which only the M_r 190,000 polypeptide appeared to be sensitive to trypsin. Molecular weights of the control proteins are indicated on the left.

ments, the binding of Fns to K562 cell polypeptides was totally inhibited (Fig 4, lane 5, pFn), and when the peptide was used to elute the bound Fns, a significant reduction in the binding was observed (Fig 4, lane 6, pFn). Control experiments with both the monoclonal (Fig 4, lane 7) and polyclonal (Fig 4, lane 8) Fn antibodies did not reveal any reaction with the K562 cells.

Surface labeling experiments with the neuraminidase-galactose oxidase-sodium borohydride method revealed that there are a few major surface-exposed glycoproteins in K562 cells, with M_r 190,000, 185,000, 120,000, 80,000 to 70,000, and 50,000 (Fig 5, lane 1). Trypsinization of the cells selectively abolished the M_r 190,000 polypeptide (Fig 5, lane 2). Octylglucoside extraction appeared to remove, apparently quantitatively, all the high M_r polypeptides from surface-labeled K562 cells (Fig 6, lanes 1 to 3). Affinity binding experiments revealed that from the octylglucoside extracts, the M_r 190,000/185,000 polypeptides specifically bound to the GRGDSPC-thiol-Sepharose (Fig 6, lane 4), and their binding could be inhibited when the RGDS peptide was included in the reaction mixture (Fig 6, lane 5).

DISCUSSION

In a series of studies¹⁰⁻¹² Lodish and his coworkers have suggested that murine erythroid cell precursors and erythroleukemia (MEL) cells specifically adhere to Fn and that this process would also play a role in normal erythropoiesis.

In this study K562 human erythroleukemia cells, which have many characteristics of early erythroid precursor cells,¹³⁻¹⁵ also displayed a specific adhesion capacity to Fn

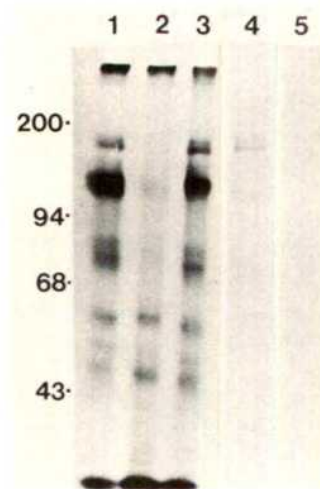


Fig 6. Autoradiogram of SDS-PAGE-separated polypeptides of surface-labeled K562 cells (lane 1), octylglucoside extract of the surface-labeled cells (lane 3), and surface polypeptides remaining in the detergent extracted cells (lane 2). Note that all the high M_r polypeptides appeared to be solubilized in octylglucoside (compare lane 3 to lanes 1 and 2). The 190,000/185,000 doublet of polypeptides bound specifically to the GRGDSPC-thiol-Sepharose (lane 4), and the binding could be inhibited by including the RGDS synthetic peptide in the reaction mixture (lane 5). Molecular weights of the control proteins are indicated on the left.

but not to, eg, laminin. The adhesion process could also be quantitatively inhibited by the tetrapeptide RGDS, like that of many other cells.²⁶⁻²⁸ However, unlike in fibroblastoid cells,² the Fn-binding property of the K562 cells appeared to be trypsin-sensitive. Furthermore, in direct overlay assays a distinct M_r 190,000 binding protein could be revealed in these cells and such a polypeptide selectively bound also to GRGDSPC-Sepharose from octylglucoside extracts of surface-labeled K562 cells.

These results appear to differ from those recently reported on MEL cells,¹¹ which express a major M_r 140,000 Fn-binding protein. This may reflect differences between human and murine erythroleukemia cells, which is evident also in their response to various differentiation-inducing agents.³² Furthermore, multiple interactions between external Fn and cell membrane elements have been recently proposed also in fibroblasts.³³⁻³⁶ Affinity binding experiments with the same Sepharose-coupled synthetic heptapeptide as used in our experiments suggested to Pierschbacher and Ruoslahti²⁸ that the peptide has too low an affinity for the Fn-receptor of fibroblasts and cannot be therefore used in its isolation. Our experiments on fibroblasts showed the same (unpublished), and thus further suggest that both the Fn-binding proteins as well as their affinities for Fns may differ in different cells, although they appear to recognize the same RGD-sequence in Fns.³⁷ Interestingly, our affinity binding experiments revealed a doublet of M_r 190,000 and M_r 185,000 polypeptides, whereas the overlay assays with ¹²⁵I-Fn and with unlabeled Fns only showed a single M_r 190,000 Fn-binding polypeptide. As only the M_r 190,000 polypeptide appeared to be trypsin-sensitive, it may be that in K562 cells the Fn-receptor is a complex of two polypeptides of which only M_r

190,000 polypeptide is capable of recognition of intact Fn. However, when comparing the apparent relative mobilities of putative Fn-receptor proteins from various sources, the recent results of Tamkun et al.³⁸ bear importance: they found that cDNA for the M_r 140,000 fibroblast Fn-receptor codes for an M_r 89,000 polypeptide. Therefore, even major variations in molecular weights may be explained by differences in, eg, glycosylation. For the M_r 190,000 Fn-binding polypeptide of the K562 cells, this remains to be elucidated.

The present results also indicate that human erythroleukemia cells are heterogenous in their response to external Fn, which may be related to different states of differentiation of the cells.³² Thus, while a portion of the K562 cells displayed a spreading capacity to Fn, many of the cells failed to do that.

Many earlier studies have suggested that in fibroblastoid cells there would be some kind of association of the pericellular Fn and the cytoplasmic actomyosin system.³⁹ In fibroblastoid cells, Fn appears to specifically induce fibrillar actin organization in some culture conditions.^{23,36,40} In the K562 cells the spreading process appeared to involve also an

emergence of the actin-microfilaments, which led even to the formation of stress fibers and vinculin-containing adhesion plaques in some of the cells. This suggests that in K562 cells, there are all the actin-associated proteins involved in the formation of stress fibers.⁴¹

The present results show that K562 erythroleukemia cells display an ability to adhere and spread on Fn and that the process is apparently mediated by a distinct receptor-protein and leads to a reorganization of the actomyosin system. The receptor protein appears to be lost in more differentiated cells, suggesting a transient nature for the adhesion process. Further studies are, however, needed to elucidate whether such a receptor-mediated adhesion of human erythroid cells on Fn would participate also in vivo in the regulation of normal or pathologic erythropoiesis.

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Monoclonal Antibody 1F5 (Anti-CD20) Serotherapy of Human B Cell Lymphomas

By Oliver W. Press, Frederick Appelbaum, Jeffrey A. Ledbetter, Paul J. Martin, Joyce Zarling, Pamela Kidd, and E. Donnell Thomas

Four patients with refractory malignant B cell lymphomas were treated with continuous intravenous (IV) infusions of murine monoclonal antibody (MoAb) 1F5 (anti-CD20) over five to ten days. Dose-dependent levels of free serum 1F5 were detected in all patients. Two patients had circulating tumor cells and in both cases 90% of malignant cells were eliminated from the blood stream within four hours of initiation of serotherapy. Antigenic modulation did not occur, and sustained reduction of circulating tumor cells was observed throughout the duration of the infusions. Serial bone marrow aspirations and lymph node biopsies were examined by immunoperoxidase and immunofluorescence techniques to ascertain MoAb penetration into extravascular sites. High doses (100 to 800 mg/m²/d and high serum 1F5 levels (13 to 190 µg/mL) were required to

coat tumor cells in these compartments in contrast to the low doses that were adequate for depletion of circulating cells. Clinical response appeared to correlate with dose of MoAb administered with progressive disease (52 mg), stable disease (104 mg), minor response (1,032 mg), and partial response (2,380 mg) observed in consecutive patients. The patient treated with the highest 1F5 dose achieved a 90% reduction in evaluable lymph node disease, but the duration of this remission was brief (six weeks). This study demonstrates that high doses of 1F5 can be administered to patients with negligible toxicity by continuous infusion and that clinical responses can be obtained in patients given >1 g of unmodified antibody over a ten-day period.

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MONOCLONAL ANTIBODY (MoAb) serotherapy of malignancy represents a theoretically attractive, potentially nontoxic approach for the treatment of neoplastic disease.¹⁻³ Preliminary animal experimentation has demonstrated both the effectiveness and limitations of MoAbs that recognize tumor-associated antigens in preventing growth of murine hematologic malignancies.^{4,5} Early human trials have shown that infusion of antibodies recognizing lymphoid cell differentiation antigens is a well-tolerated therapy capable of coating tumor cells and causing tumor regression in some patients.⁶⁻⁸ However, the antitumor effectiveness of MoAbs has been limited by the presence of circulating free antigen, antigenic modulation, development of human antimouse antibodies (HAMA), emergence of antigen-negative tumor cell variants, and the inadequacy of host effector cell mechanisms.^{5,8-11}

Here we present findings in four patients with B cell lymphomas treated with a murine IgG2a MoAb (1F5) chosen to avoid many of the previously encountered obstacles. MoAb 1F5 recognizes a 35,000 dalton antigen (Bp35,

CD20) present on the surface of normal and malignant B cells¹² that is not shed from the cell surface (unpublished observations), does not modulate in response to MoAb binding, and does not bind to any other normal tissues. Consequently, prolonged continuous MoAb 1F5 therapy can be administered without inducing the unresponsiveness to therapy that has necessitated intermittent bolus therapy in previous trials.⁸⁻¹¹ We have administered 1F5 by continuous intravenous (IV) infusion (52 to 2,380 mg over five to ten days) to determine toxicity, kinetics, penetration to extravascular tissues, and efficacy. Our studies have shown 1F5 to be a minimally toxic therapy capable of depleting circulating tumor cells at low doses and lymph node tumor cells at high doses. However, responses were transient, suggesting that antibodies conjugated to toxins or radioisotopes might afford more lasting clinical benefit than unmodified antibody.

MATERIALS AND METHODS

Antibody preparation. Murine MoAb 1F5 (IgG2a) was produced in BALB/c mice and purified as previously described.¹³ Antibody 1F5, along with the B1 antibody,¹⁵ has been assigned to the CD20 (anti-Bp35) cluster group by the Second International Workshop on Human Leukocyte Differentiation Antigens.¹⁴ The reactivity of antibody 1F5 with normal and malignant B cells has previously been reported.^{13,16,17} MoAb 1F5 was purified from ascites by saturated ammonium sulfate precipitation followed by diethyl aminoethyl (DEAE)-Sephacryl (Pharmacia, Piscataway, NJ) column chromatography.¹⁶ Testing of the purified antibody by Microbiological Associates (Bethesda, MD) has shown it to be free of bacterial, viral, or endotoxin contamination. Preclinical testing in two macaques (*M. fascicularis*) injected with 1F5 IV showed that this antibody was capable of eliminating circulating B cells and penetrating lymph nodes without causing any acute toxicity (J. Ledbetter, unpublished observation, 1983). A battery of normal human autopsy tissues was screened for reactivity with antibody 1F5 by an indirect immunoperoxidase method. No reactivity was seen with any tissue except those known to be rich in B lymphocytes (tonsils, lymph nodes, spleen). Tissues failing to bind 1F5 included heart, thyroid, adrenal, lung, muscle, kidney, testis, skin, colon, breast, and brain.

Patient selection. Adult patients with histologically confirmed B cell lymphomas shown by immunoperoxidase or immunofluorescence techniques to be reactive with the 1F5 antibody were eligible

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for this study if they had failed previous conventional therapy (chemotherapy and/or radiotherapy), if they had normal renal and hepatic function (creatinine <2.0 mg/dL, bilirubin <1.5 mg/dL), had evaluable disease, had not received any other treatment for $\geq$ four weeks, had no other active medical problems, and signed an informed consent approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Study design. Prestudy blood, marrow, and lymph node specimens were obtained and analyzed by conventional histopathology and by an indirect immunoperoxidase method (Vectastain, Vector Laboratories, Raritan, NJ) for evidence of tissue involvement with tumor cells capable of binding 1F5. Cell suspensions of these tissues were analyzed by two-color flow cytometry using a panel of fluorescein and phycoerythrin antibody conjugates¹³ to determine the baseline immunologic phenotypes of the resident cell populations (see below). Intradermal skin testing with 10 μ g of antibody 1F5 in 0.1 mL of normal saline was performed, and no hypersensitivity responses were observed. Allopurinol (300 mg/d) was given throughout antibody administration. A bolus loading dose was given over one to two hours IV to rapidly achieve steady state serum antibody levels. The loading dose was calculated from the following equation: Loading dose = $1.4 \times$ elimination half-time (in days) $\times$ daily maintenance dose.¹⁸ Preclinical studies of murine anti-CD20 antibody infusions in nonhuman primates (Ledbetter, unpublished data) and clinical trials of murine MoAbs administered to patients with graft-v-host disease (GVHD)¹⁹ suggested an elimination half-time of 1.2 days, and this figure was used in calculating the loading doses. Patients were assigned a predetermined maintenance antibody dose that was diluted in 500 mL normal saline and administered by continuous IV infusion for five to ten days. (Patient 1 had premature discontinuation of his infusion after five days due to rapidly progressive lymphoma.) The maintenance antibody doses administered to the patients are summarized in Table 1. The dose escalation range was chosen to progress from safe low doses (5 mg/m²/d) known to be well tolerated for other MoAbs^{6,7,9,10,19} to high doses (400 to 800 mg/m²/d), which we felt were more likely to result in good tissue penetration. We initially planned to escalate doses between patients. However, because of the absence of toxicity, poor penetration of low doses of antibody into patients 1 and 2, and slow patient accrual, doses were escalated progressively in patients 3 and 4 (from 10 mg/m²/d to 800 mg/m²/d) to achieve high circulating antibody levels that we felt would be more likely to achieve extravascular tissue penetration.

Table 1. Summary of Patient Characteristics

Patient	1	2	3	4
Age/Sex	42/M	64/M	63/M	45/M
Type of Lymphoma	DML	WDLL	DPDL	DHL
Stage	IVB	IVA	IVA	IVB
Total Dose of 1F5	52.4 mg	104.8 mg	1,032 mg	2,380 mg
Duration of Therapy	4.5 days	10 days	10 days	7 days
Response	Progression	Stable Disease	Minor Response	Partial Response

Abbreviations: DML, diffuse mixed small and large cell lymphoma; WDLL, diffuse well-differentiated lymphocytic lymphoma (small lymphocytic, working formulation); DPDL, diffuse, poorly differentiated lymphocytic lymphoma (diffuse small cleaved cell, working formulation); DHL, diffuse "histiocytic" lymphoma (diffuse large cell lymphoma, working formulation).

Patient monitoring. Pretreatment tests included a history and physical examination, relevant radiographic studies and computed tomographic scans, chemistry batteries, uric acid levels, complete blood cell counts (CBCs) and differentials, prothrombin time, partial thromboplastin time, serum complement levels (CH50, C4, C3), immune complex levels (C1Q binding assay), urinalysis, ECG, and cell surface marker analysis. Patients were examined twice daily during antibody infusion. Serial serum specimens for 1F5 levels and antimouse antibody levels, blood counts, chemistries, and blood specimens for surface marker studies were obtained four hours after initiation of 1F5 therapy and daily thereafter. Patients were discharged at the termination of antibody infusions. Blood samples were obtained on an outpatient basis for the above tests on days 1, 2, 7, and 21 after cessation of therapy and monthly thereafter. Serum complement and immunoglobulin levels were tested pretreatment and on days 1, 5, and 10 and then at roughly monthly intervals for six months.

Response criteria. Standard response criteria were employed as follows: Complete response-disappearance of all measurable and evaluable disease; Partial response: reduction by $\geq 50\%$ of leukemic cell counts and $\geq 50\%$ reduction in the size of a measurable lesion, and no increase in the size of any measurable or evaluable lesions or appearance of new lesions; Stable disease: Less than a partial response without an increase of $>25\%$ in leukemic cell count and $<25\%$ increase in any measurable lesion. Progression: Increase in leukemic cell count ($>25\%$), appearance of new lesions, or an increase of 25% or greater in any measurable lesion.

Measurement of free 1F5 and human antimouse antibody. Serum 1F5 levels and human antimouse antibody levels (HAMA) were measured by solid phase competitive inhibition radioimmunoassay (RIA) as previously described.¹⁹

Detection of cell-bound 1F5. Assessment of tumor cell coating by infused antibody 1F5 was accomplished on serial specimens of peripheral blood, bone marrow, and lymph nodes by indirect immunoperoxidase and immunofluorescence techniques. Peripheral blood and bone marrow mononuclear cells were obtained by Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, MD) density gradient centrifugation. Lymph node biopsies were divided in thirds: one portion was minced into a single cell suspension, another part was fixed in formalin for routine histologic staining, and another portion was frozen in liquid nitrogen for immunohistologic staining. Lymph node frozen sections were fixed to gelatin-coated glass slides and stained with rabbit antimouse immunoglobulin (Vectastain, Vector Laboratories, Raritan, NJ) using an indirect avidin-biotin technique. Pretreatment biopsies served as controls.

Cell suspensions of blood, bone marrow, and lymph nodes from antibody-treated patients were examined by flow cytometry (FACS IV, Becton Dickinson, Sunnyvale, CA) for the presence of surface 1F5 by using fluorescein-conjugated goat antimouse immunoglobulin (FITC-GAM1g; TAGO, Burlingame, CA). The mean fluorescence intensity of cells stained with FITC-GAM1g was compared to the intensity of cells incubated with excess 1F5 in vitro before staining to assess the saturation of binding sites in vivo. Relative CD20 surface antigen density was estimated for normal and malignant B cells by measuring the mean fluorescence intensity of cells stained in vitro with saturating quantities of fluorescein-conjugated anti-CD20 antibody after correcting for nonspecific fluorescence with a control reagent.^{13,17}

Tumor cell surface antigen phenotypes were determined by both immunofluorescence and immunoperoxidase methods using peroxidase, fluorescein, or phycoerythrin conjugates of MoAbs 10.2 (anti-CD5), HB10a (anti-DR), G1-4 or 3E10 (antikappa), and 2C3 (anti- μ) as previously described.^{13,17} Serial monitoring of these tumor cell markers demonstrated that CD20-negative tumor cells were not

generated during 1F5 therapy by antigenic modulation (data not shown).

CASE HISTORIES

Patient 1 was a 42-year-old man with stage IVB diffuse, mixed, small and large cell lymphoma who presented in 1982 with fever, diffuse lymphadenopathy, and hepatosplenomegaly. Previous therapy included CHOP (cyclophosphamide, adriamycin, vincristine, and prednisone) chemotherapy, intrathecal methotrexate, whole brain irradiation, splenectomy, sequential upper and lower hemibody irradiation, and four cycles of bleomycin, cytosine arabinoside, vincristine, procarbazine, and prednisone. He was referred for 1F5 therapy in July, 1984 because of refractory disease. He did not respond to low-dose 1F5 and was taken off study after five days of infusion because of progressive bone marrow (BM) and liver replacement with tumor. Salvage CHOP chemotherapy was given, but the patient died of progressive lymphoma on March 16, 1985.

Patient 2 is a 64-year-old man with stage IVA diffuse, small, lymphocytic lymphoma diagnosed by lymph node (LN) and BM biopsy in 1976. He received multiple chemotherapeutic regimens (CVP, CHOP, chlorambucil, and CCNU, etoposide, and methotrexate) with partial responses. He was referred for 1F5 serotherapy in December 1984, 11 months after his last course of chemotherapy. He did not respond to low dose 1F5 but had stable disease that did not require therapy until the summer of 1985 when he was begun on bleomycin, etoposide, BCNU, and Decadron, to which he remains partially responsive.

Patient 3 was a 63-year-old man with stage IVA diffuse, small, cleaved-cell lymphoma involving lymph nodes, marrow, and spleen. Previous therapy included splenectomy, chlorambucil, and CVP. He was referred for 1F5 therapy in December 1984, one month after his last cycle of CVP because of the development of refractory disease with rapidly progressive adenopathy and lymphocytosis ($>30,000$ cells/ μ L). He showed a minor response to intermediate dose 1F5 therapy. ProMACE/MOPP chemotherapy was given in January and February 1985 without response. A partial response occurred after therapy with high-dose cytosine arabinoside, but the patient died with marrow aplasia in July 1985.

Patient 4 was a 45-year-old man with sclerosing, diffuse large-cell lymphoma presenting in January 1983 with bowel and lymph node involvement. Therapy included eight cycles of CHOP, intrathecal methotrexate, involved field abdominal radiation, prophylactic cranial irradiation, and allogeneic marrow transplantation (in March 1984). He was referred for 1F5 therapy because of refractory lymphoma in late October 1985. He had been on dexamethasone (4 mg/d) for many months as symptomatic therapy for myalgias, and this was continued during serotherapy. After treatment with 1F5 there was a partial response that lasted six weeks. He then redeveloped progressive lymphoma and refused further treatment. He expired on December 21, 1985.

RESULTS

Serum 1F5 levels. Circulating free antibody levels were detectable by RIA in all patients throughout the period of infusion (Fig 1). Patients 1 and 2 received MoAb doses of 5 mg/ m^2 /d and consistently had 1F5 serum concentrations of 0.3 to 1.0 μ g/mL. Patients 3 and 4 received escalating antibody doses and had corresponding increases in 1F5 levels. For comparable antibody doses the patients with circulating antigen-positive tumor cells (1 and 3) had lower serum levels of 1F5 than the patients who did not have significant numbers of circulating malignant lymphocytes (2 and 4, see Fig 1), probably reflecting the effect of antibody

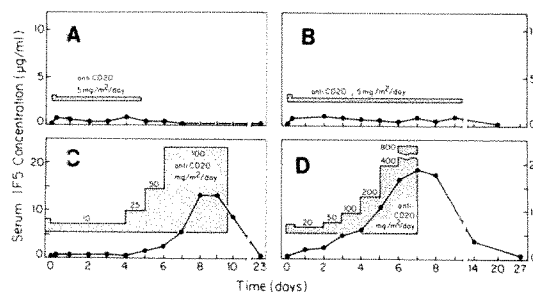


Fig 1. Serum 1F5 concentrations in patients receiving continuous infusions of antibody. MoAb 1F5 concentrations were determined by solid phase radioimmunoassay. The doses of 1F5 infused in each patient are indicated by the superimposed stippled bars. (A), patient 1; (B), patient 2; (C), patient 3; (D), patient 4.

binding to tumor cells. Antibody remained detectable in the serum for as long as three weeks after termination of infusion in patient 4 (peak concentration 190 μ g/mL). However, 1F5 levels fell into the undetectable range within three days of termination of infusion in patients 1 and 2 (peak levels 1 μ g/mL) and within two weeks in patient 3 (peak level 13.4 μ g/mL). Rough estimates of the serum elimination half-times were calculated to be 24 hours for patient 1 (from 0.93 μ g/mL to .22 μ g/mL in the 48 hours after termination of infusion), 42 hours for patient 3 (from 13.4 μ g/mL to 9.0 μ g/mL in 24 hours), and 52 hours in patient 4 (from 179 to 35.6 μ g/mL over five days). Data for patient 2 were insufficient for estimation of a serum half-life. These elimination half-times are in good agreement with previous studies of murine anti-CD20 antibodies in nonhuman primates (J. Ledbetter, unpublished results) and studies of murine anti-T cell antibodies in patients with GVHD.¹⁹

CD20 antigen density on tumor cells. Table 2 summarizes the relative Bp35 surface antigen densities on patient lymphoid cells from blood, bone marrow, and lymph nodes as determined by direct immunofluorescent analysis.^{13,17} The density of this antigen on normal B lymphocyte populations is also listed for comparison. Patients 1 and 4 had CD20 densities on their malignant cells comparable to those seen on normal, resting B lymphocytes (eg, peripheral blood B cells and tonsil mantle zone B cells¹³). Patient 3 had a much higher surface antigen density on his lymphoma cells, comparable to that observed on normal, activated B cells (tonsil germinal center cells¹³). Patient 2 had a very low Bp35 antigen density on his lymph node and bone marrow tumor cells with a mean fluorescence intensity only 3.5 times higher than control cell populations lacking the antigen. (This degree of staining was unequivocally greater than control, however.) Patients 2 and 4 had negligible numbers of circulating tumor cells morphologically, confirming the negligible staining with FITC-1F5 seen by immunofluorescence (nearly all circulating lymphocytes were T cells in these patients). Of interest, the bone marrow of patient 4 was grossly involved with tumor but failed to bind FITC-1F5, suggesting that an antigen-negative tumor cell variant was responsible for infiltration of this tissue. With this single exception, the different sites of lymphomatous involvement within a given patient showed similar CD20 antigen densi-

Table 2. Relative CD20 Antigen Densities on Normal and Malignant Lymphoid Cells

Cell Type	Relative Antigen Density*
Normal Tissues	
1. Peripheral Blood T cells	2†
2. Peripheral Blood B cells	82
3. Tonsil Mantle Zone B cells	70
4. Tonsil Germinal Center B cells	256
Lymphoma Patients	
1. Patient 1‡	
a. Blood lymphocytes	96
2. Patient 2	
a. Bone marrow	7
b. Lymph node cells	7
3. Patient 3	
a. Blood lymphocytes	301
b. Bone marrow cells	301
c. Lymph node cells	235
4. Patient 4	
a. Blood lymphocytes (uninvolved)	2
b. Bone marrow cells	2
c. Lymph node cells	84

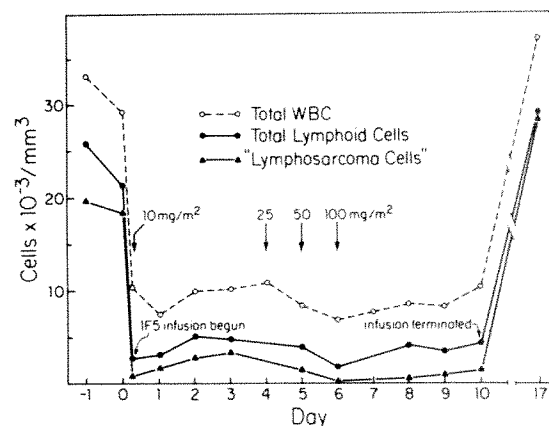
*Expressed as the linear channel number of the mean fluorescence intensity measured on a FACS IV cell sorter for cells stained with saturating concentrations of fluorescein-conjugated anti-CD20 antibody by the method of Ledbetter and Clark.¹³

†Negative control (unstained) cells also showed a mean fluorescence intensity of 2.

‡Patient 1 had an unascrable marrow and no accessible adenopathy, so immunofluorescent studies were done solely on circulating malignant cells.

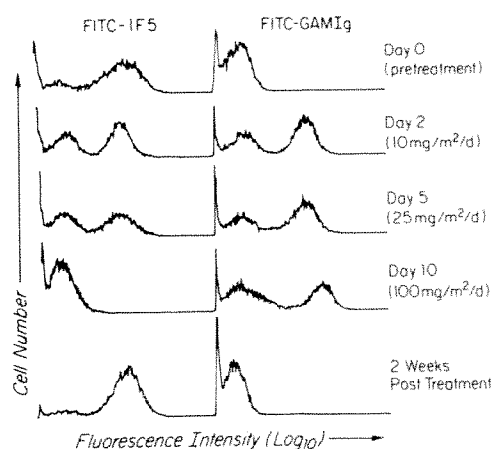
ties. No definitive conclusion regarding the clinical responsiveness of tumors bearing different surface CD20 densities is possible because of the small number of patients treated and variable antibody doses administered.

Effects of 1F5 on peripheral blood lymphocytes. Two patients (1 and 3) had appreciable numbers of circulating malignant cells. In both patients antibody administration resulted in an immediate decrease in the number of circulating tumor cells (assessed by morphological criteria and by surface immunologic phenotypes). Patient 1 had an 86% decline in the number of blood lymphoma cells (from $1.27 \times 10^3/\mu\text{L}$ to $0.18 \times 10^3/\mu\text{L}$) within four hours of institution of 1F5 therapy. Patient 3 had a 91% decrement in circulating tumor cells (from $18.21 \times 10^3/\mu\text{L}$ to $1.61 \times 10^3/\mu\text{L}$) in the same brief time interval (Fig 2). These effects were obtained with low doses of antibody in both patients ($5 \text{ mg}/\text{m}^2$ and $10 \text{ mg}/\text{m}^2$, respectively) and were sustained throughout the entire period of infusion (five and ten days). Flow cytometry of circulating PBL stained with FITC-GAM1g demonstrated saturation of 1F5 antibody binding sites on tumor cells in both patients (although complete saturation in patient 3 was only achieved at the higher dose of $100 \text{ mg}/\text{m}^2/\text{d}$, Fig 3). Serial tumor cell surface-antigen phenotyping (using two-color immunofluorescence with reagents recognizing other tumor-associated antigens [see Materials and Methods]) demonstrated that antigenic modulation did not occur (data not shown). In both patients termination of 1F5 therapy was accompanied by a rapid reappearance of

**Fig 2. Depletion of circulating lymphoma cells in patient 3 during infusion of MoAb 1F5.**

circulating tumor cells that reached pretreatment levels within two to three days (Fig 2).

Effects of 1F5 on bone marrow tumor cells. Patients 1, 2, and 3 had evaluable marrow involvement with lymphoma. In patients 1 and 2, antibody doses of $5 \text{ mg}/\text{m}^2/\text{d}$ were not sufficient for saturation of 1F5 antibody binding sites on tumor cells in the marrow. Patient 3 received escalating doses of 1F5 in conjunction with serial marrow aspirations to estimate the amount of antibody required for coating of tumor cells in the marrow. Serial fluorescence histograms (Fig 4) clearly showed that an antibody dose of $10 \text{ mg}/\text{m}^2/\text{d}$ was insufficient (4% saturation of Bp35 binding sites) but that $100 \text{ mg}/\text{m}^2/\text{d}$ could produce significant coating (61% saturation of Bp35 binding sites) of marrow tumor cells.

**Fig 3. In vivo labeling of circulating tumor cells with 1F5 antibody in patient 3 as assessed by serial flow cytometry of peripheral blood lymphocytes with FITC-GAM1g (to detect mouse antibody 1F5 bound to tumor cells in vivo) and FITC-1F5 (to detect unoccupied Bp35 [CD20] binding sites). Pretreatment PBL stained brightly with FITC-1F5 because of abundant free CD20 sites on circulating lymphoma cells. Serial histograms on days 2, 5, and 10 revealed coating of PBL with 1F5 (detected with FITC-GAM1g). Saturation of binding sites is shown on day 10 by absence of unoccupied Bp35 receptors capable of binding FITC-1F5. By two weeks posttreatment, bound murine MoAb was no longer detectable on PBL.**

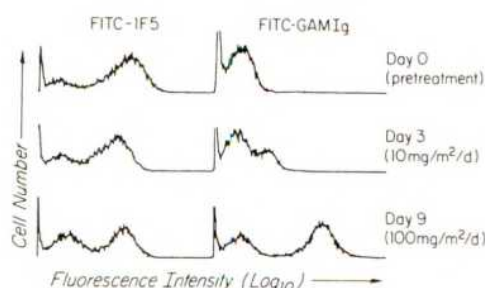


Fig 4. Penetration of MoAb 1F5 into bone marrow of patient 3 during serotherapy as assessed by flow cytometry of mononuclear cells from BM aspirates using FITC-GAMlg (to detect cell-bound 1F5) and FITC-1F5 (to detect unbound Bp35 sites).

Regression of marrow lymphoma was not seen in any of these three patients.

The marrow of patient 4 was unusual in that it appeared to contain tumor cells that did not express the Bp 35 antigen. Although marrow aspirates and biopsies contained unequivocal large cell lymphoma, no tumor cells reactive with FITC-1F5 were detected by flow cytometry. In contrast, tumor cells in cervical and inguinal lymph nodes had the same morphology as the cells in the marrow but reacted strongly with antibody 1F5 as assessed by both immunoperoxidase and immunofluorescence techniques (see Table 2). As would be anticipated, infusion of antibody 1F5 had no effect on the antigen-negative tumor cells in the marrow of this patient.

Effects of 1F5 on lymph nodes. Patients 2, 3, and 4 had evaluable adenopathy that was biopsied before treatment and on the last day of antibody infusion. Immunoperoxidase and immunofluorescent analyses showed no penetration of antibody 1F5 into the nodes of patient 2 (who received 105 mg over ten days). There was minor perivascular penetration detectable only by immunoperoxidase methods in patient 3 (1,032 mg over ten days). In contrast, significant coating of tumor cells detectable by both immunoperoxidase and immunofluorescence (Figs 5 and 6) was present in patient 4 (2,380 mg over seven days) with 69% saturation of available binding sites. In vitro studies showed that an ambient 1F5 antibody concentration of 24 μ g/mL was necessary to achieve 69% saturation of cell-surface binding sites. Since the serum 1F5 concentration in patient 4 at the time of his lymph node biopsy was approximately 190 μ g/mL, we estimate that a 1F5 antibody gradient of 8:1 existed between serum and lymph node interstitial fluid.

No clinical response was observed in the nodes of patient 2. Some inguinal nodes regressed by 25% in patient 3, but most lymph nodes were unaffected. There was marked regression of all nodes in patient 4 with a calculated >90% reduction in tumor burden (Fig 7). Of note, the diminution of LN size did not begin until day 5 of antibody infusion, and progressive node shrinkage continued for three weeks after cessation of 1F5 infusion. The response duration was brief, however, with regrowth of LN occurring six weeks after therapy.

Overall clinical response. Patient 1 had diminution of

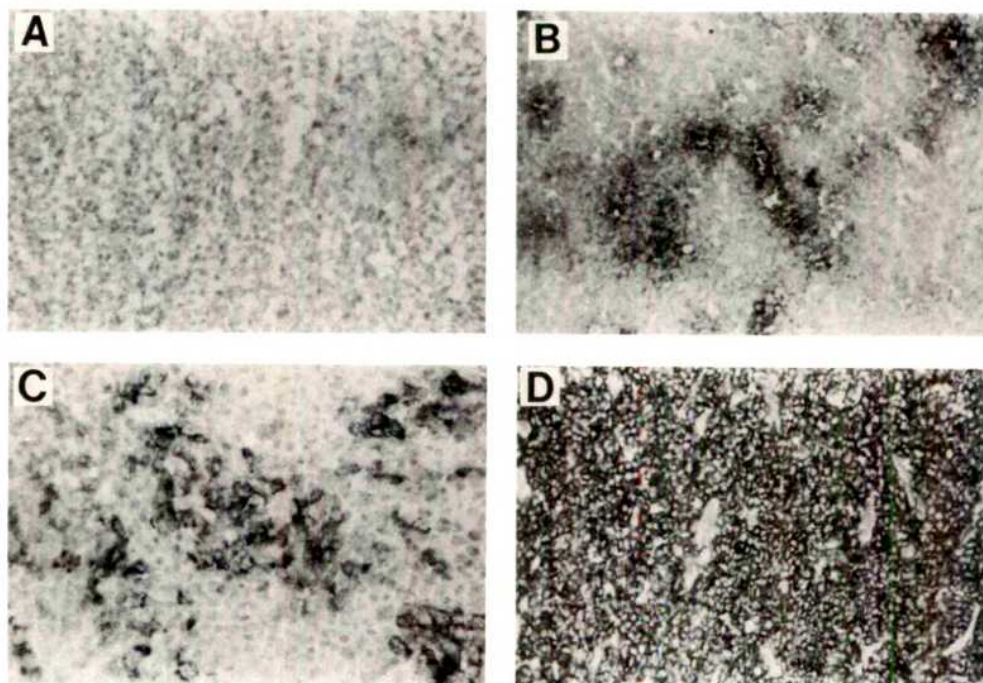


Fig 5. Indirect immunoperoxidase staining of lymph node frozen sections with GAMlg to detect *in vivo* labeling of tumor cells during murine MoAb 1F5 serotherapy (original magnification $\times 250$). (A) Patient 2: LN biopsy performed on day 10 of therapy while receiving 5 mg/m²/d of antibody 1F5. Negligible staining indicates absence of penetration of LN by antibody at this dose. (B) Patient 3: LN biopsy performed on day 9 of therapy while receiving 100 mg/m²/d of 1F5. Staining of tumor cells in perivascular locations is present. (C) Patient 4: LN biopsy performed on day 10 of therapy while receiving 800 mg/m²/d of 1F5. Peroxidase staining is appreciable at this dose, although the distribution remains heterogeneous. (D) Saturation of 1F5 binding sites in patient 3 by *in vitro* incubation of an LN section with excess 1F5 antibody. The section shown in this figure was from the same LN biopsy depicted in Fig 4B.

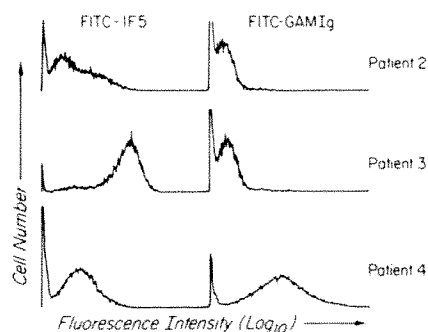


Fig 6. Analysis of 1F5 penetration into LN during serotherapy assessed by flow cytometry of LN suspensions. FITC-GAM1g was used to detect *in vivo* coating of tumor cells with antibody 1F5. Patient 2 had only a small subpopulation of LN cells possessing the Bp35 antigen (shown with FITC-1F5). No penetration of 1F5 into LN could be shown with FITC-GAM1g. Virtually all LN cells in patient 3 possessed the Bp35 antigen and stained intensely with FITC-1F5, but poor *in vivo* penetration of 1F5 had occurred as shown by absence of FITC-GAM1g staining. (Immunoperoxidase staining of LN sections was capable of demonstrating some penetration of 1F5 into this LN, however [see Fig 5B]). Patient 4 had significant penetration of 1F5 into LN as shown by staining with FITC-GAM1g.

circulating tumor cells, but progressive liver and marrow lymphoma required premature termination of 1F5 therapy and institution of salvage chemotherapy. Patient 2 had no response of his evaluable marrow or LN disease. Patient 3 had a minor response consisting of transient 90% reduction of circulating tumor cells and 25% shrinkage of some but not all LN but no response in the marrow. Patient 4 had a partial response consisting of 90% reduction of all evaluable LN.

Toxicity. No clinically significant toxicity was observed in any of our patients. Patients 1 and 4 had asymptomatic, intermittent, low-grade fever (38 to 39 °C) lasting six days and two hours, respectively. Transient decrements of platelet and neutrophil counts to 50% to 75% of baseline levels were observed in all four patients. These changes were rapidly reversible in all cases except patient 1, where progressive cytopenias were clearly due to marrow replacement with tumor. In the other three instances, the blood counts stabilized after one to two days and often demonstrated some recovery, even before discontinuation of antibody infusion. No bleeding or infectious episodes occurred during antibody treatment. No allergic, pulmonary, renal, hepatic, or cutaneous sequelae occurred. Renal function as measured by 24-hour creatinine clearance was unchanged after completion of antibody therapy. Complement consumption was observed in patients 1 (40% reduction of CH₅₀ and C4 levels) and 3 (97% reduction of CH₅₀, 94% reduction of C4 levels), but circulating immune complexes could not be detected at any time in any patient.

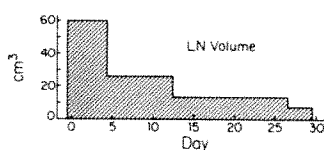


Fig 7. Reduction in tumor burden in patient 4. A 90% reduction in LN volume was observed over a four-week period.

Effects of 1F5 on normal B cells. Attempts to monitor numerical changes and functional alterations of normal B cells in patients receiving 1F5 serotherapy (by assessing proliferative responses to polyclonal B cell mitogens and *in vitro* Epstein-Barr virus (EBV)-induced immunoglobulin synthesis) were precluded by the extremely low numbers of normal B cells that could be harvested from these patients following antibody infusion. The paucity of B cells was due to two factors: baseline deficiency of normal B lymphocytes (a common feature of advanced, refractory B cell malignancies) and depletion of normal as well as malignant B cells by 1F5 therapy (which has also been documented in normal nonhuman primates infused with anti-CD20 antibodies, [Jeffrey Ledbetter, unpublished results]). Quantitative immunoglobulin levels were carefully monitored in all patients before, during, and for up to five months after 1F5 therapy. Baseline hypogammaglobulinemia was present in patients 1 and 3, but no patient demonstrated a decline in any subclass of immunoglobulin consequent to 1F5 therapy.

Human antimouse antibody levels. IgM HAMA were undetectable by RIA in all patients. Low levels of IgG HAMA (2 × pretreatment control levels) became detectable five months after serotherapy in patient 1 only.

DISCUSSION

This report summarizes our findings in four patients with refractory malignant B cell lymphomas treated with MoAb 1F5 (anti-CD20) by continuous IV infusion for five to ten days. Our study differs from previous serotherapy trials of hematologic malignancies by employing an antibody directed against a nonmodulating antigen. This feature allowed us to maintain continuous high serum antibody levels without inducing the tumor refractoriness generally encountered with modulating antigens.^{8-11,20} The continuous infusion mode of administration allowed delivery of very high doses of antibody (up to 800 mg/m²/d in patient 4) without the significant pulmonary toxicity that is often observed following bolus injection of high MoAb doses.^{8,10,21} Serial kinetic measurements revealed a dose-dependent relationship between the amount of antibody infused and the concentration of free MoAb in the blood stream. We found that even low doses of 1F5 (5 to 10 mg/m²/d) were capable of depleting circulating tumor cells from the blood stream analogous to observations made using murine MoAbs T101 (for chronic lymphocytic leukemia) and J5 (for acute lymphocytic leukemia).^{9-11,20} However, in contrast to the studies with modulating antibodies T101 and J5 given by prolonged or repeated administration, the responses induced by the nonmodulating 1F5 were sustained throughout the duration of the infusion (five to ten days).

Although small doses of 1F5 sufficed to deplete circulating tumor cells, penetration of antibody into extravascular sites such as bone marrow and lymph nodes proved to be much more problematic. Intravenous administration of 400 to 800 mg/m²/d was required to achieve 69% saturation of binding sites on lymph node tumor cells. Even at these doses the intranodal distribution of antibody was heterogeneous. The immunoperoxidase staining patterns observed in LNs

suggested passive diffusion of antibody down a concentration gradient from small blood vessels into the LN parenchyma. The clinical responsiveness observed appeared to correlate with 1F5 dose administered, peak-serum MoAb concentration achieved, and degree of extravascular tissue penetration obtained. A total dose of 52.4 mg was associated with progressive disease (patient 1), 104.8 mg resulted in stable disease in patient 2, 1,032 mg caused a minor response in patient 3, and 2,380 mg produced a partial response in patient 4.

These observations have been made on a very small group of patients, each of whom had a different type of B cell lymphoma. The degree to which our findings might be applicable to other lymphoma patients is unclear, since each patient has a different tumor burden and distribution, different numbers of circulating malignant cells acting to absorb infused MoAb, and different surface densities of the Bp35 antigen. Consequently, the dose levels and kinetic data found in our patients can only serve as a rough guideline for the management of other patients with this antibody. Generalizations from our findings with 1F5 to other MoAbs should be made with caution in light of recent studies demonstrating dramatic, unpredictable kinetic and functional differences among different antibodies recognizing the same antigen.²²

The toxicity seen in these four patients was insignificant. Minor fever and moderate cytopenias were the only adverse effects observed despite administration of massive doses of antibody 1F5 to patients 3 and 4. Since neither platelets nor neutrophils express the Bp35 antigen or label with FITC-1F5, the exact mechanism for the decrement in neutrophil and platelet counts is uncertain. It is of interest that similar decrements in blood counts have also been observed in patients treated with anti-idiotypic antibodies.⁸ Small quantities of antibody might be absorbed via Fc receptors to these cells, which may then be removed from the circulation by the reticuloendothelial system. Although the development of HAMA has been a major problem in some reported series,^{8,23} antimouse antibodies were detected in only one of our four patients, and in this patient they did not appear until five months after 1F5 therapy was completed. These findings are in accord with other studies demonstrating that patients with B cell malignancies undergoing monoclonal serotherapy seldom develop HAMA, whereas patients with T cell malignancies or solid tumors receiving similar treatment often develop antimouse antibodies.^{24,25}

Previous trials of MoAb serotherapy have also generally encountered minimal toxicity.³ The major adverse events described to date involved anaphylactoid reactions in patients with large circulating tumor cell burdens and/or high circulating antigen levels given high doses of antibody by rapid bolus injection.^{8,10,21} These episodes have been ascribed to pulmonary leukostasis resulting from sequestration of antibody-coated tumor cells in the pulmonary vasculature leading to wheezing, dyspnea, and hypotension. The absence of circulating antigen and the prolonged duration of antibody administration in our trial were mitigating factors that probably helped avoid these untoward sequelae in our patients.

The relative merits of continuous infusion of MoAbs

compared with intermittent bolus therapy remain debatable. For many antibodies continuous infusion is not feasible because of antigenic modulation. In such circumstances intermittent therapy is necessary to allow regeneration of cell surface antigen. The bolus method is less cumbersome than continuous infusion and achieves higher peak MoAb concentrations for equivalent doses. Whether the maintenance of uniform high-circulating antibody levels and reduction in toxicity achievable with continuous infusion are sufficiently advantageous to offset the inconveniences remains unanswered. Nevertheless, maintenance of steady state antibody levels in this trial has afforded an advantageous setting for kinetic measurements and for the assessment of the serum concentrations required for MoAb penetration into the extravascular space.

The mechanisms by which unmodified MoAbs might cause elimination of tumor cells *in vivo* remain controversial. Most workers currently view antibody-dependent cellular cytotoxicity and reticuloendothelial system phagocytosis of MoAb-coated cells as the most likely processes involved.^{3,26} Murine MoAbs (including 1F5) fix human complement poorly *in vitro*, and consequently complement-mediated tumor cell lysis is not thought to be of major significance *in vivo*. The significant consumption of complement in two of our four patients was unexpected and suggests a possible role for complement in eliminating tumor cells.

The short, incomplete responses obtained with serotherapy using unmodified MoAbs have been of minimal clinical benefit (with the notable exception of the patient described by Miller et al).⁷ Consequently, innovative MoAb administration schedules, testing of new antibodies, and administration of antibody conjugates will be necessary if the promise of monoclonal serotherapy is to be realized. Badger et al have already convincingly demonstrated cures of lymphomas in mice treated with radioiodinated MoAbs in a setting in which unmodified MoAbs were ineffective.²⁷ Recent clinical reports of responses in patients with Hodgkin's disease or hepatoma treated with radioiodinated antiferritin antibodies suggest that this approach will also be useful in man.^{28,29} Radiolabeled MoAbs can potentially kill not only the tumor cells to which they bind but could also kill neighboring cells that do not bind antibody by virtue of poor tissue penetration, antigenic modulation, or somatic mutation ("antigen-negative variants"). Toxin-antibody conjugates are similarly promising,³⁰ although antigens such as CD20, which are not endocytosed after ligand binding,³¹ might not be good targets for this approach, since immunotoxin internalization is generally required for cell killing.³² The findings of our current pilot study should assist in the rational design of subsequent trials employing such radiolabeled or toxin-conjugated immunotoxins.

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Comparative Thrombolytic Properties of Single-Chain Forms of Urokinase-Type Plasminogen Activator

By David C. Stump, Jean M. Stassen, Eddy Demarsin, and Désiré Collen

The specific thrombolytic properties of urokinase and three molecular forms of single-chain urokinase-type plasminogen activator (scu-PA) were compared in a human plasma milieu *in vitro* and in an experimental thrombosis model in rabbits. These scu-PA molecules included M_r 54,000 scu-PA from human urine (urinary scu-PA), scu-PA from conditioned media of a human lung adenocarcinoma cell line (CALU-3, ATCC, HTB-55) (cellular scu-PA) and an M_r 32,000 proteolytic derivative of cellular scu-PA (scu-PA-32k). All four molecular forms induced significant lysis of a ^{125}I -labeled human plasma clot immersed in citrated human plasma at concentrations between 50 and 200 IU/mL. None of the four showed absolute fibrin-specificity, but at equivalent lytic dose the three single-chain forms appeared to cause less fibrinogen degradation and α_2 -antiplasmin consumption than two-chain urokinase. In addition, the fibrinolytic potential of the three single-chain forms was largely maintained during pre-incubation in plasma for up to 48 hours whereas that of urokinase was

completely inhibited. Intravenous (IV) infusion of cellular scu-PA or scu-PA-32k into rabbits with a ^{125}I -labeled thrombus in the jugular vein caused significant dose-dependent lysis at concentrations ranging from 8,700 to 35,000 and from 9,000 to 36,000 IU/kg respectively. Clot lysis was accompanied by minor α_2 -antiplasmin consumption or fibrinogen breakdown. In contrast, urokinase induced lysis at doses between 20,000 and 200,000 IU/kg, but at higher doses was associated with significant systemic activation of the fibrinolytic system. It is concluded that scu-PA obtained from CALU-3 cell cultures has identical thrombolytic properties to that obtained from urine. In addition, the scu-PA-32k proteolytic derivative has the same fibrin-specific thrombolytic properties as the intact molecule. Cellular scu-PA and scu-PA-32k may therefore constitute more readily available alternatives for clot-selective thrombolytic therapy in man.

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CONVERSION of plasminogen to plasmin is the key event occurring during physiologic fibrinolysis. Most efficiently it occurs at or near the fibrin surface where rapid inhibition of the generated plasmin does not take place.¹ Urokinase, obtained from human urine,² activates both circulating and fibrin-bound plasminogen similarly. As a result, it induces fibrinolysis but only in association with the so-called "lytic state," characterized by consumption of α_2 -antiplasmin and fibrinogen breakdown, which may predispose to bleeding.³ In contrast, fibrin-specific thrombolysis is now possible with tissue-type plasminogen activator (t-PA), which efficiently activates plasminogen only in the vicinity of a blood clot⁴ as the result of its specific affinity for fibrin.⁵ Its promise as a fibrin-specific thrombolytic-in-man agent has now been confirmed.⁶

More recently, a single-chain molecular form of urokinase has also been recognized as a potential fibrin-specific thrombolytic agent.⁷⁻¹² These molecular forms of urokinase have been designated pro-urokinase or preferably single-chain urokinase-type plasminogen activator (scu-PA).¹³ According to some studies, scu-PA activates plasminogen with high

affinity ($K_m < 1 \mu\text{mol/L}$), even in the absence of fibrin,^{14,15} but this activity is inhibited by plasma. However, with the presence of fibrin in plasma, scu-PA regains its plasminogen-activating potential, though not via direct binding to fibrin. To date scu-PA has not become widely available for use in biochemical and clinical studies. The most readily available biological source of scu-PA, human urine, indeed contains such small amounts ($< 15 \mu\text{g/L}$)^{16,17} that it is impractical for use in large-scale purification. Moreover, to date, urinary scu-PA, though fully characterized physicochemically and kinetically,¹⁷ has not been shown to be stable in plasma or to have clot-selective fibrinolytic properties in plasma.

A more widely available and enriched secondary source (CALU-3 cells) of scu-PA has been identified and a straightforward method for its purification developed.¹⁸ This has also led to the identification of a novel proteolytically modified M_r 32,000 form of scu-PA,¹⁹ which has similar kinetic properties as intact scu-PA. The purpose of the present study was to compare the thrombolytic properties of urinary scu-PA, cellular scu-PA, and scu-PA-32k with those of urokinase.

MATERIALS AND METHODS

Human urinary scu-PA was purified as described¹⁷ with a specific activity of 51,000 IU/mg as determined by calibration against the International Reference Preparation for Urokinase (66/96) obtained from Dr P.J. Gaffney, National Institute for Biological Standards and Control, London, UK. Cellular scu-PA was purified from conditioned media of cultured human lung adenocarcinoma cells (CALU-3, ATCC, HTB-55)¹⁸ with a specific activity of 35,000 IU/mg. M_r 32,000 scu-PA (scu-PA-32k) with a specific activity of 71,000 IU/mg was obtained from this same cell line as described elsewhere.¹⁵ Prior to use all three forms of scu-PA were freed of contaminating urokinase by chromatography on benzamidine-sepharose,²⁰ which reduced the urokinase content to less than 1%. Urinary urokinase (Winkinase) was a gift from Dr E. Murano, Bureau of Biologics, Bethesda, MD.

Human fibrinogen²¹ and thrombin²² were prepared by the methods described. Fibrinogen labeling with ^{125}I (Amersham Research

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Products, Amersham, UK) was performed with the Enzymobead Reagent (Biorad, Richmond, CA). Desalting and purification were accomplished by filtration over a 10 mL column of Sephadex G25 (Pharmacia, Uppsala, Sweden) and a 0.7×60 cm column of Ultrogel AcA 44 Ultrogel (LKB, Bromma, Sweden) respectively.

In vitro lysis of human plasma clots. The relative fibrinolytic and fibrinogenolytic properties of the different forms of scu-PA and of urokinase were determined in an in vitro system consisting of a ^{125}I -labeled human plasma clot immersed in citrated human plasma, as previously described.²³ The ^{125}I -labeled plasma clots were added to 2.5 mL aliquots of pooled plasma, counted, and warmed to 37°C in a water bath. Fifty microliters of plasminogen activator were then added. At one-hour intervals, 250 μL of plasma were removed, the ^{125}I content measured, and the percent lysis determined from the solubilized radioisotope. Controls without added plasminogen activator were determined at each hourly sampling point and always were less than 5% to 10% of total releasable radioactivity over the four-hour experimental period. Plasma fibrinogen levels were determined immediately on each hourly sample,²⁴ and α_2 -antiplasmin levels were later measured on frozen samples.²⁵ Reported results are means of at least three separate experiments.

The stability of the plasminogen activators in plasma was measured in the following way. Mixtures were incubated at room temperature for varying periods of time with concentrations of plasminogen activator chosen to give at least 50% lysis within four hours. After pre-incubation, freshly prepared ^{125}I -labeled plasma clots were added to the mixtures that were placed at 37°C . Lysis rates were measured and calculated as above on duplicate samples.

Thrombolysis in a rabbit jugular vein thrombosis model. The in vivo thrombolytic properties of cellular scu-PA and scu-PA-32k were compared to those of urokinase in the rabbit jugular vein thrombosis model previously described.²⁶ In the present study ^{125}I -labeled jugular vein thrombi were aged for 30 minutes, then the plasminogen activator was administered as a 10% bolus injection, followed by continuous infusion of the remaining 90% dose over four hours via a contralateral marginal ear vein. Thrombolysis was quantitated 30 minutes after the end of the infusion by counting the residual radioactivity in the jugular vein segment. Blood samples of 1 mL were drawn into 0.01 mol/L citrate and used for determinations of residual fibrinogen and α_2 -antiplasmin levels.

RESULTS

The dose response of fibrinolysis in human plasma in vitro of the three forms of scu-PA and of urokinase is shown in Fig 1. Urinary scu-PA induced significant clot lysis at a concentration of 200 IU/mL in two to three hours (Fig 1A). At a twofold higher concentration, more rapid lysis occurred, 35% within one hour and 95% within two hours. No significant lysis was observed over the four-hour observation period at concentrations below 100 IU/mL. Similar dose-related fibrinolytic responses were also seen with cellular scu-PA (Fig 1B) and scu-PA-32k (Fig 1C), with significant lysis occurring with 100 IU/mL or more and to lysis with 50 IU/mL or less. Lysis progressed with time with all three molecular forms of scu-PA. When lysis induced by urokinase was quantitated (Fig 1D), initially a more rapid and more potent effect was seen. The addition of only 50 IU/mL induced 50% lysis within one hour. This rapid initial lysis was proportional to the amount of urokinase added, but in contrast to that observed with the scu-PA forms, the subsequent lysis rates decreased with time. With urokinase, lysis thus occurred predominantly within the first two hours.

The relative fibrin-specificities of the urokinase-type plas-

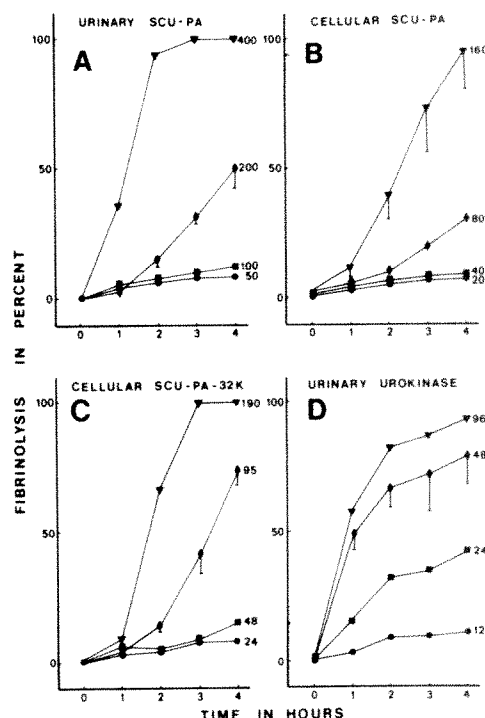


Fig 1. Lysis of human plasma clots labeled with ^{125}I fibrin following addition of (A) urinary scu-PA (●—●: 50 IU/mL; ■—■: 100 IU/mL; ◆—◆: 200 IU/mL; ▼—▼: 400 IU/mL). (B) cellular scu-PA (●—●: 20 IU/mL; ■—■: 40 IU/mL; ◆—◆: 80 IU/mL; ▼—▼: 160 IU/mL). (C) scu-PA-32k (●—●: 12 IU/mL; ■—■: 24 IU/mL; ◆—◆: 48 IU/mL; ▼—▼: 95 IU/mL; ▼—▼: 190 IU/mL). (D) urokinase (●—●: 12 IU/mL; ■—■: 24 IU/mL; ◆—◆: 48 IU/mL; ▼—▼: 96 IU/mL). Control values without added plasminogen activator were: at one hour: $3.7\% \pm 1.3\%$; at two hours: $5.6\% \pm 1.7\%$; at three hours: $6.7\% \pm 2.3\%$; at four hours: $7.0\% \pm 3.1\%$. Each data point represents the mean value of at least three separate experiments $\pm$ SD for one concentration of each giving significant lysis.

minogen activators (u-PAs) are shown in Fig 2. At concentrations of 200 IU/mL (urinary), 160 IU/mL (cellular), or 95 IU/mL (scu-PA-32k) 50% or more lysis was observed within four hours, all with less than 25% fibrinogen breakdown. At concentrations of 400 IU/mL of urinary scu-PA (Fig 2A) or of 200 IU/mL of scu-PA-32k (Fig 2C), which gave 100% after three hours, plasma fibrinogen levels decreased to below 25% of the baseline level. With urokinase (Fig 2D), 45% to 55% lysis occurred after one hour with both 48 and 96 IU/mL, concomitant with 30% and 70% fibrinogen degradation respectively. At a concentration of 96 IU/mL urokinase was also associated with a greater than 75% decrease in fibrinogen levels after two hours while not causing 100% lysis even after four hours. Thus, although none of the urokinase-type plasminogen activators was absolutely fibrin-specific in this in vitro model, equivalent clot lysis appeared to be accompanied by less extensive fibrinogen degradation with all three single-chain forms. Measurement of α_2 -antiplasmin levels confirmed this, with less than 50% consumption associated with 50% lysis by the three single-chain forms, while 50% lysis by urokinase was always accompanied by greater than 50% α_2 -antiplasmin consumption (not shown).

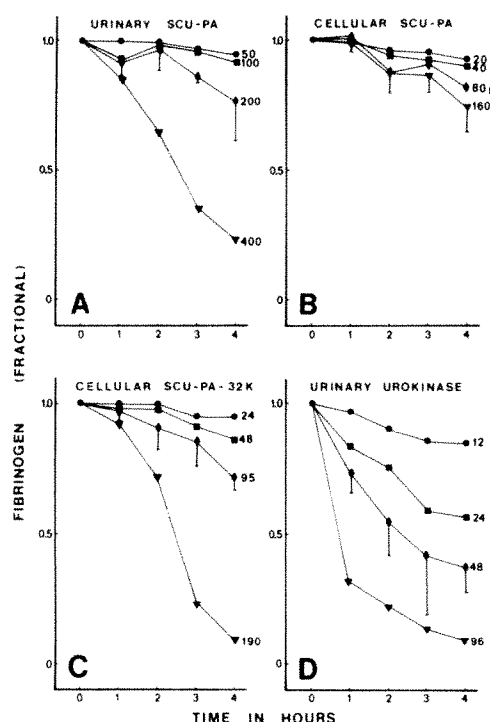


Fig 2. Plasma fibrinogen levels during lysis of human plasma clots labeled with ^{125} I-fibrin following addition of urinary scu-PA (A); cellular scu-PA (B); scu-PA-32k (C); and urokinase (D). The symbols represent the same concentrations as in Fig 1. Control fractional fibrinogen levels without added plasminogen activator were at one hour: 0.99 ± 0.025 ; at two hours: 0.92 ± 0.059 ; at three hours: 0.96 ± 0.034 ; and at four hours: 0.90 ± 0.045 . Each data point represents the mean value of at least three separate experiments $\pm$ SD for one concentration of each giving significant lysis.

The relative stability of scu-PA in plasma was illustrated by pre-incubation studies with results shown in Fig 3. Lysis of freshly prepared plasma clots was measured after pre-incubation of mixtures of plasma with each of the four forms of u-PA at room temperature. Without preincubation all three single-chain forms, at concentrations between 160 and 400 IU/mL, gave 50% lysis within 1.5 to 2.5 hours. The more

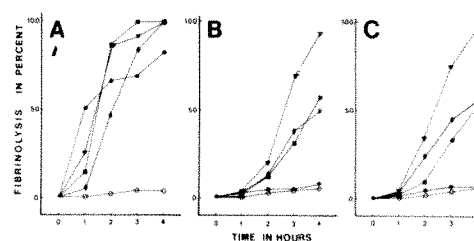


Fig 3. Lysis of freshly prepared human plasma clots labeled with ^{125} I-fibrin after pre-incubation with urinary scu-PA 400 IU/mL ($\blacksquare$); cellular scu-PA 160 IU/mL ($\blacklozenge$); scu-PA-32k 190 IU/mL ($\blacktriangledown$); urokinase 48 IU/mL ($\bullet$); and control without added plasminogen activator ($\circ$). (A) 0 hours; (B) 24 hours; (C) 48 hours. Each data point represents the mean of two experiments.

rapid initial rate for urokinase was evident by 50% lysis with 48 IU/mL at only one hour (panel A). After pre-incubation for 24 hours (panel B) and 48 hours (panel C), the potency of the single-chain forms was largely maintained, with 50% lysis still occurring within 2.5 to 4 hours. In contrast, urokinase was completely inhibited within the first 24 hours.

The thrombolytic properties and the fibrin specificity of the more readily available cellular scu-PA and scu-PA-32k were compared with those of urokinase in a rabbit jugular vein thrombosis model with results summarized in Table 1. Lysis measured 30 minutes after the end of infusion of solvent into control animals was $8.9\% \pm 0.6\%$. Dose-dependent lysis (12% to 48%) was observed for urokinase over a range of 20,000 to 200,000 IU (0.2 to 2.0 mg) per kilogram. A similar range of lysis (13% to 40%) was produced by infusion of cellular scu-PA over a concentration range of 8,700 to 35,000 IU (0.25 to 1.0 mg) per kilogram and by scu-PA-32k between 9,000 and 36,000 IU (0.13 to 0.5 mg) per kilogram. Comparable thrombolysis (20% to 25%) was obtained with 100,000 IU/kg urokinase, 17,000 IU/kg scu-PA, and 18,000 IU/kg scu-PA-32k, ie, five- to eightfold lower total doses for both forms of scu-PA than for urokinase. In addition, both forms of scu-PA failed to induce systemic fibrinolytic activation, as evidenced by normal

Table 1. Thrombolysis After Infusion of Different Molecular Forms of scu-PA or Two-Chain Urokinase in Rabbits With Jugular Vein Thrombosis

Agent	Dose (IU/kg)	n	Lysis (percent)	Recovery (percent)	Fibrinogen (percent)	α_2 -Antiplasmin (percent)
Control	—	5	8.9 ± 0.6	99 ± 1.8	101 ± 3	94 ± 4
Urokinase	20,000	3	12 ± 0.8	99 ± 1.5	101 ± 5	90 ± 6
	50,000	3	16 ± 2.3	94 ± 2.2	95 ± 5	87 ± 2
	100,000	3	22 ± 0.8	95 ± 0.9	91 ± 7	74 ± 6
	200,000	4	48 ± 4.3	93 ± 5.3	39 ± 24	42 ± 14
Cellular scu-PA	8,700	3	13 ± 1.3	94 ± 1.0	92 ± 12	92 ± 7
	17,000	6	21 ± 1.4	94 ± 1.8	99 ± 3	85 ± 3
	35,000	4	34 ± 3.8	88 ± 1.8	92 ± 2	97 ± 4
scu-PA-32K	9,000	3	15 ± 1.1	96 ± 1.0	98 ± 2	90 ± 8
	18,000	3	24 ± 1.5	94 ± 3.3	99 ± 4	95 ± 2
	36,000	3	40 ± 4.1	93 ± 3.2	90 ± 5	100 ± 2

Each figure represents the mean $\pm$ standard error of the mean of the number of experiments indicated by n.

fibrinogen and α_2 -antiplasmin levels. Lysis with urokinase occurred with less fibrin-specificity as reflected by the dose-dependent consumption of α_2 -antiplasmin. Fibrinogen degradation with urokinase was very pronounced at the highest dose (200,000 IU/kg) with plasma levels falling to 39% of baseline.

DISCUSSION

Evidence is accumulating that the single-chain form of urokinase, presently designated scu-PA,¹⁶ shows potential as a fibrin-specific thrombolytic agent. Although scu-PA isolated from its natural source, human urine, has been characterized to some extent,^{16,17} all information on its fibrin specificity in plasma *in vitro*^{8,9} or *in vivo*^{7,8,10-12} has been obtained with material isolated from secondary sources, eg, cell culture or recombinant DNA technology. In the present study we compared the specific thrombolytic properties of scu-PA derived from human urine, its natural biological source, with that of scu-PA isolated from CALU-3-conditioned cell culture media. Both forms of scu-PA do induce clot lysis with relatively higher fibrin specificity than urokinase in a human plasma milieu. In addition, both scu-PA forms demonstrate relatively higher stability in plasma than urokinase, with an approximately 50% preservation of fibrinolytic capacity after 48 hours incubation of scu-PA with plasma but total inhibition of urokinase within 24 hours. This relative stability, as previously recognized for scu-PA from other secondary natural⁸ or recombinant sources,⁹ has been ascribed to the presence of specific plasma inhibitor(s) of urokinase. Such an inhibitor has in fact now been identified in human plasma with inhibitory activity toward urokinase but not scu-PA.²⁷ This inhibition would not account for the partial reduction in fibrinolytic activity of the single-chain forms seen during pre-incubation, though some loss of activity after prolonged room temperature exposure is perhaps not surprising.

From these observations two extrapolations can be made. First, since scu-PA circulating in blood²⁸ is most likely identical to that isolated from urine, it may as such contribute to physiologic fibrin-specific thrombolysis via its relatively unique fibrinolytic properties. Second, scu-PA from cellular origin, specifically isolated from CALU-3-conditioned cell culture media, having biological properties identical to those of urinary scu-PA, may constitute a valid and

more practical alternative for the study of the biochemical and biological properties of scu-PA.

In addition, the *in vitro* and *in vivo* thrombolytic properties of scu-PA-32k, a previously unrecognized molecular form of scu-PA, were compared with that of its well-characterized M_r 54,000 counterpart. Though structurally different, both forms induced very similar clot lysis, relatively distinct from that of urokinase. Their molar-specific fibrinolytic activities, as measured on fibrin plates, were identical, although their thrombolytic potency *in vivo* was higher than that of urokinase, as contrasting with the higher *in vitro* potency of urokinase. This discrepancy may well be related to species differences between the plasma systems utilized, especially with the known lack of sensitivity of the rabbit to urokinase.²⁹

While our results indicate that the moiety(ies) responsible for the fibrin specificity of scu-PA are not dependent upon the NH_2 -terminal and kringle-containing portions of the M_r 54,000 protein, which are indeed absent in scu-PA-32k,¹⁹ the exact structures responsible for its unique action remain, however, to be more precisely defined. Since scu-PA appears to act more via a high affinity for its natural substrate plasminogen than via a specific affinity for fibrin, it is perhaps not surprising that the domains responsible for lysine (fibrin) binding could be deleted without any impact on the fibrinolytic capacity of the enzyme. In fact, scu-PA-32k does display the same high affinity for plasminogen ($K_m = 0.9 \mu\text{mol/L}$) as M_r 54,000 scu-PA, a property not present in either molecule following conversion to two-chain u-PA by cleavage of the Lys 158-Ile 159 peptide bond.¹⁹ In any case, scu-PA-32k, a low M_r variant of scu-PA with identical thrombolytic properties, may constitute a more practical alternative to the native high M_r form, particularly for the large-scale production of this thrombolytic agent by recombinant DNA technology. As such it may offer significant potential for future development.

In summary, it is concluded that scu-PA derived from its natural biological source, human urine, has distinctive fibrinolytic properties from urokinase. Furthermore, the more readily available scu-PA, obtained from CALU-3 cell structures, has identical thrombolytic properties to the material of urinary origin. Thus, scu-PA and scu-PA-32k, a proteolytic derivative of scu-PA, may constitute valid alternatives for future physiologic and therapeutic studies in man.

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Hematologic Engraftment and Immune Reconstitution Posttransplantation With Anti-B1 Purged Autologous Bone Marrow

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Hematologic engraftment and immune reconstitution were examined in patients who received cyclophosphamide and total body irradiation therapy followed by infusion of autologous bone marrow purged with anti-B1 monoclonal antibody (MoAb) and complement as therapy for non-Hodgkin's lymphoma. Hematologic engraftment was prompt with return of $\geq 0.5 \times 10^3/\mu\text{L}$ granulocytes and $\geq 2 \times 10^4/\mu\text{L}$ platelets at a median of 26 and 29 days posttransplant, respectively. Immunologic reconstitution, in contrast, was prolonged. Normal numbers of circulating B cells were consistently noted by five months posttransplant, whereas return of normal immunoglobulin levels in some patients did not occur for one year. Normal numbers of T cells were

evident within the first month posttransplant, but a reversed T4:T8 ratio persisted in some patients up to three years. In vitro responses of either B cells to triggers of activation or of T cells to mitogens and antigens were not normal for at least three months posttransplant. Natural killer (NK) cells predominated early after transplant and may demonstrate cytotoxicity against tumor cells. Our studies demonstrate that transplantation with anti-B1 purged autologous bone marrow results in complete hematologic and delayed immunologic engraftment. No significant acute or chronic clinical toxicities have been observed.

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HIGH-DOSE chemoradiotherapy and bone marrow support now have become an important treatment modality for patients with relapsed non-Hodgkin's lymphoma (NHL).¹ Although earlier studies employed syngeneic^{2,3} or allogeneic⁴⁻⁷ bone marrow to rescue the aplasia induced by ablative therapy, most recent trials now employ autologous marrow.⁸⁻²¹ This approach has demonstrated high efficacy with complete responses approaching 90% and minimal toxicity compared with allogeneic transplantation. A more general application of autologous bone marrow transplantation (ABMT) for NHL has been limited by lymphomatous infiltration of the marrow.²²⁻²⁶ To overcome this obstacle, several approaches to purge the marrow of tumor cells have been suggested. These include techniques to remove tumor cells on the basis of their density, osmotic fragility, sensitivity to chemotherapy, and/or expression of tumor-associated cell surface antigens.²⁷ However, the success of these techniques, either alone or in combination, is dependent upon the removal of tumor cells from the marrow without damaging hematopoietic stem cells.

The eradication of lymphoma cells from the bone marrow of patients with relapsed B cell NHL has been one of our major interests during the past 3½ years. We have treated minimally involved bone marrows of patients with relapsed B cell NHL with the monoclonal antibody (MoAb) anti-B1 and rabbit complement in an attempt to render the marrow tumor free.^{1,13,21} The anti-B1 MoAb defines a nonglycosylated phosphoprotein of 35kD whose expression within the hematopoietic system appears to be B cell restricted.²⁸⁻³⁰ This antigen appears on the B cell surface prior to the cytoplasmic μ positive pre-B cell stage and is lost from the cell surface as cells become plasma cells. Detailed in vitro experiments demonstrated that the B1 antigen is not expressed on either myeloid or erythroid stem cells.³¹ Moreover, treatment with anti-B1 MoAb and complement in vitro eliminates clonogenic Burkitt's cells from normal human bone marrow.³²

Although hematologic engraftment in our patients who received anti-B1-purged ABMT has been rapid and complete, it is equally important to demonstrate immunologic engraftment. Relapsed lymphoma patients rescued with untreated autologous bone marrow have demonstrated rapid resolution of granulocytopenia and thrombocytopenia by

three to four weeks post-ABMT.^{8-11,18} However, the reconstitution of immune function following ABMT for lymphoma has been less well studied. We have therefore attempted to examine both hematologic and immunologic engraftment of patients who have undergone ABMT after purging of marrow tumor cells with anti-B1 MoAb and complement.

MATERIALS AND METHODS

Hematologic Engraftment

Peripheral blood samples obtained from 24 patients who underwent autologous bone marrow transplantation therapy are included in this analysis: 23 patients with NHL and a single patient with chronic lymphocytic leukemia. Prior to in vitro marrow purging, patients were treated with conventional chemotherapy, radiation therapy, and/or surgery to a minimal tumor burden and a histologically uninvolved bone marrow with <5% cells expressing B1. To date all patients have attained a complete clinical response and have achieved complete hematologic engraftment with minimal acute and chronic clinical toxicity. The day posttransplant, when circulating white blood cells appeared, as well as the interval to achievement of $\geq 0.5 \times 10^3$ granulocytes/ μL and a stable untransfused platelet count of at least $2 \times 10^4/\mu\text{L}$ served as monitors of hematologic

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engraftment. The duration of hospitalization posttransplant was also recorded.

Patient Samples

After appropriate human protection validation and informed consent, patient samples were obtained for study. Peripheral blood samples were obtained prior to and at various intervals posttransplantation. Mononuclear cells were obtained from peripheral blood (PBMCs) by Ficoll-Hypaque density sedimentation³³ for phenotypic and functional analysis. Sufficient numbers of cells were not available for every time point; in particular, detailed data were not collected for the earliest patients undergoing this treatment. In addition, no analysis was performed beyond the time of relapse for nine patients.

Phenotypic Analysis

A panel of MoAbs specific for B and T cells, monocytes, and natural killer (NK) cells was used in this study (Table 1). PBMCs were assayed for reactivity with the MoAbs by indirect immunofluorescence staining and flow cytometric analysis as described previously.³⁴ Antibody-coated cells were enumerated by flow cytometric analysis using an EPICS V cell sorter (Coulter Electronics, Hialeah, FL). For each sample 10,000 cells were analyzed using a log amplifier.

Functional Assays

Peripheral blood samples (60 mL) were obtained from patients and at least one normal donor at the time of each functional assay.

Stimulation of B Cells

B cell proliferation assays were performed as previously described.⁴² B cell-enriched preparations were made from PBMCs by E rosette depletion whenever numbers of cells permitted. Because it is extremely difficult to purify B cells from peripheral blood to homogeneity using both depletion as well as enrichment techniques,⁴² we chose anti-u, which is a stimulus for B cell but not for T cell proliferation.⁴²⁻⁴⁴ Cells were resuspended at 5×10^5 cells/mL in RPMI/10% fetal calf serum, and 100- μ L aliquots were dispensed in 96-well round-bottomed tissue culture plates (Costar, Cambridge, MA). Cells were cultured in the presence of either rabbit anti-u chain-conjugated polyacrylamide beads (30 μ g/mL, wt/vol) (Immunobead, BioRad, Richmond, CA) or media to a final culture volume of 200 μ L/well. To measure proliferation, some cultures were pulsed at 48 hours with 0.2 μ Ci/well (1 Ci = 3.7×10^{10} Bq) of

tritiated thymidine ($[^3\text{H}]\text{TdR}$), were harvested 15 hours later onto glass filters, and were counted on a liquid scintillation counter (Packard Tri-Carb 4530, Downers Grove, IL). A stimulation index (proliferation noted to a given stimulus/proliferation noted to media alone) ≥ 2.0 was judged to be significant. B cells from normal donors were stimulated at the time of each patient assay and served as controls.

Stimulation of T Cells

PBMCs were resuspended at 1×10^6 cells/mL in RPMI/10% human AB serum, and 100 μ L aliquots were dispensed in 96-well, round-bottomed tissue culture plates (Costar). Cells were cultured in the presence of either media alone or stimuli at the following final concentrations: phytohemagglutinin (PHA) 1:5 and 1:10; concanavalin A (Con A) 1:20 and 1:40; mumps 1:20; tetanus toxoid 1:10; and *Candida albicans* 1:50. For allogeneic mixed-lymphocyte reactions, Epstein-Barr virus (EBV)-transformed Laz 388 cells were irradiated to 5,000 R, resuspended at 1×10^6 /mL, and 100- μ L aliquots dispensed into 96-well round-bottomed plates containing equal numbers of patient PBMCs. Twenty microliters of T cell growth factor containing media (TCGF-CM) was also added to wells already containing patient cells and/or media, PHA, or Laz 388 cells. In all cases cells were cultured at 37 °C in 5% CO₂ and pulsed with 0.2 μ Ci/well (1 Ci = 3.7×10^{10} Bq) of tritiated thymidine ($[^3\text{H}]\text{TdR}$), harvested 18 hours later, and counted as described above. The increment in proliferation noted to the individual stimulus compared to media was calculated for both the patient and for the normal control examined on the same day. The patient response to any given stimulus was judged to be significant if it was $\geq 40\%$ of proliferation of normal cells cultured with the identical stimulus. Finally, proliferation noted to PHA or Laz 388 was compared to that noted when cells were cultured with these stimuli supplemented with TCGF-CM. The addition of TCGF-CM to PHA was judged to significantly enhance proliferation if proliferation to TCGF-CM and PHA/proliferation to PHA ≥ 1.5 . The TCGF-CM used in these studies was obtained as previously described.⁴⁵

Cytotoxicity (Natural Killer Cell) Assays

Cytotoxicity assays were performed according to a standard chromium release method previously described.⁴⁵ Assays were performed at various effector:target ratios using 5,000 K562 target cells/well. Significant NK cell function was $>5\%$ killing of K562, as evidenced by chromium release assay.

RESULTS

Hematologic Engraftment

A median of 5.1 (range 2.0 to 8.5×10^7) autologous bone marrow cells/kg were reinfused. Marrow engraftment occurred in all patients with the first evidence of white blood cell recovery generally by days 10 to 12 and 0.5×10^3 granulocytes/ μ L at a median of 26 (range 12 to 45) days after transplant. A stable nontransfused platelet count of at least 2×10^4 / μ L was attained at a median of 29 (range 14 to 137) days. Finally, duration of hospitalization was a median of 33 (range 20 to 58) days posttransplant. Eighty-four percent, 10%, and 6% of deglycerolized frozen red blood cells were transfused in the first, second, and third months posttransplant, respectively. Eighty percent of platelets were transfused in the first month, 19.5% in the second month, and only 0.5% after eight weeks posttransplant.

Table 1. Reactivity of Monoclonal Antibodies

Monoclonal Antibody	CD	Lineage Restriction	Molecular Weight (kilodaltons)	Reference
Anti-B4	19	Pan-B	95	34
Anti-B1	20	Pan-B	35	29, 30
Anti-B2	21	Limited-B (EBV/C3d receptor)	140	35
Anti-T3	3	Mature T (Part of T3-Ti T cell receptor/complex)	19-29	36
Anti-T11	2	Pan-T (Alternative pathway of T cell activation)	50	36
Anti-T4	4	Mature helper/inducer T cell	55	37
Anti-T8	8	Mature cytotoxic/suppressor T cells	32-33	38
Anti-Mo2	w14	Monocytes	55	39
Anti-NKH1	—	Large granular lymphocytes	200-220	40, 41

Table 2. Cell Surface Phenotype of Mononuclear Cells Posttransplant

Weeks Posttransplant	No. Patients Normal*/No. Patients Tested											
	Pre	2	3	4	8	12-16	20-24	28-36	40-52	56-64	68-72	76-145
B cells	6/14	0/10	1/14	4/15	6/16	5/9	7/7	6/6	5/5	4/4	5/5	3†/3
T cells	3/14	10/10	9/14	10/16	11/16	5/9	1/7	4/6	2/5	2/4	0/5	2/3
T4/T8	4/14	0/10	2/13	2/16	0/16	2/9	0/6	0/6	0/5	1/4	1/4	0/3
NK cells	4/10	6/7	7/11	9/13	8/13	3/9	5/7	4/6	3/5	0/4	2/5	2/3
Monocytes	12/14	1/8	5/13	11/15	10/15	7/9	5/6	6/6	4/5	4/4	4/4	3/3

*Normal numbers of cells were as follows: 5% to 10% PBMCs reactive with anti-B1, anti-B2, or anti-B4 (B cells); 60% to 75% PBMCs reactive with anti-T3 and/or anti-T11 (T cells); 10% PBMCs reactive with anti-901 or anti-NH₁ (NK cells); 10% to 15% PBMCs reactive with anti-Mo2 (monocytes). Normal T4:T8 ratio is 1.5 to 2.5.

†Two of the three patients have been repeatedly tested during this interval.

Immunologic Engraftment

Phenotypic Analysis

A panel of MoAbs directed at B and T cells, monocytes, and natural killer (NK) cells (Table 1) was utilized in an indirect immunofluorescence assay to monitor the phenotype of PBMCs as bone marrow engraftment and reconstitution of peripheral blood leukocytes proceeded (Table 2).

B cells. Normal numbers of B lymphocytes, as reflected by reactivity with anti-B1, anti-B2, and/or anti-B4 MoAbs, were present in six of 14 patients examined before transplantation. Cells bearing these antigens were totally absent in ten patients at two weeks and present in only one of 14 patients at three weeks post-ABMT. At one month post-ABMT, 27% (four of 15) patients had normal B cells: two of these patients had multiply relapsed lymphoma, and two patients were less heavily pretreated since they were transplanted earlier in the course of their disease. By eight to 16 weeks posttransplant, approximately 50% of patients demonstrated normal percentages of B1⁺, B2⁺, and/or B4⁺ cells, and at ≥20 weeks post-ABMT all patients studied had normal numbers of B lymphocytes.

T cells. Total T cells were enumerated by reactivity with anti-T3 and/or anti-T11 MoAbs. Only 21% (three of 14) patients had normal numbers of T cells prior to ABMT. Within two to four weeks of reinfusion, ≥60% of the mononuclear cells expressed T3 and/or T11. Many of the T11⁺ or T3⁺ cells noted early after transplant are large granular

lymphocytes that co-express cell surface NKH1. As B cells and monocytes reappear and increase in number beginning at three to four weeks post-ABMT, the percentage of T11⁺ cells decreases. The T4:T8 ratio (normal 1.5 to 2.5) remains abnormal in some patients beyond one year post-ABMT due to both a decrease in T4⁺ helper/inducer cells as well as an increase in T8⁺ cytotoxic/suppressor cells.

NK cells. Natural killer (NK) cells, which express the NKH1 antigen, were ≥10% PBMCs in only 40% (four of 11) of patients examined prior to ABMT. However, within the first month following ABMT, the overwhelming majority of patients had increased numbers of cells (up to one third of PBMCs) expressing the NK phenotype. In some cases this increased percentage of cells with NK cell phenotype persisted to one year posttransplant.

Monocytes. Monocytes (Mo2⁺) composed ≥15% PBMCs in 86% of patients tested prior to transplant, in five of 13 patients at three weeks post-ABMT, and in ≥2/3 of patients tested beyond one month following ABMT.

Functional Analyses

B cell proliferation. B cell-enriched populations were obtained at various intervals post-ABMT and examined for their responsiveness to anti-u, a known B cell specific mitogen (Table 3). Two of four patients tested prior to transplant demonstrated significant proliferation [stimulation index (SI) ≥2.0] to anti-u bound to polyacrylamide

Table 3. B Cell Stimulation*

Response	No. Patients Responding/No. Patients Tested			
	Pre	Weeks Posttransplant		
		0-4	4-8	12-116†
Stimulation Index to anti-u ≥ 2 (range)	2 (0.9-12.9) 4	0 (0.99-1.17) 9	0 (0.88-1.16) 8	9† (1.51-12.2) 10

*B cell-enriched populations were suspended at 5×10^5 cells/mL in RPMI/10% fetal calf serum, and 100-μL aliquots were dispensed in 96-well round-bottomed tissue culture plates. Cells were cultured in the presence of either rabbit anti-u chain-conjugated polyacrylamide beads (30 μg/mL, wt/vol) or media. Cultures were pulsed 48 hours later with tritiated thymidine ($[^3\text{H}]\text{TdR}$) and harvested and counted 15 hours later. Counts per minute of $^3\text{H-TdR}$ in cultures with media alone were as follows: pre, 161-500; zero to four weeks, 320-947; four to eight weeks, 413-1126; and 12 to 116 weeks, 192-877.

†Five patients were repeatedly tested during this time interval.

beads. Of nine and eight patients tested at zero to four and four to eight weeks post-ABMT respectively, none demonstrated significant proliferation. By 12 to 116 weeks post-ABMT, B cells from nine of ten patients again proliferated to anti-u.

Serum immunoglobulin (Ig) levels. Serum IgG, IgA, and IgM levels were normal in the majority of patients tested prior to and at four weeks post-ABMT ($\geq 6/13$ and $\geq 6/10$ patients, respectively) (Table 4). By eight to 20 weeks post-ABMT, a minority of patients had normal Ig levels. At 24 to 52 weeks post-ABMT, $\geq 50\%$ of patients had reconstituted normal serum Ig levels, and nearly all patients tested one year post-ABMT had normal levels of IgG, IgA, and IgM.

T cell proliferation. PBMCs were cultured in the presence of mitogens, antigens, or growth factors known to trigger T lymphocyte proliferation. Responses were considered to be positive if the proliferation to a given T cell stimulus was $\geq 40\%$ of the increment noted in a normal donor's cells cultured under identical conditions. As can be seen in Table 5, approximately 50% of patients examined prior to transplant responded to phytohemagglutinin (PHA) or concanavalin A (Con A). Responses to either mitogen were absent for a two-month period post-ABMT. In four of ten patients tested at 12 to 116 weeks posttransplant, T cell responses were again noted to either mitogen. Antigen-specific assays for the stimulation of T cell proliferation included mumps, tetanus toxoid, and *Candida albicans*. As was true for both PHA and Con A, only 50% of patients examined prior to ABMT responded to these antigens. Within the first month posttransplant, residual responsiveness was noted in 22% to 44% of patients tested. However, responses were largely absent by the second month posttransplant, and only two of ten patients tested at ≥ 12 weeks responded to these antigens.

T cell growth factor containing medium (TCGF-CM) obtained by stimulating PBMCs with PHA, phorbol myristate acetate, and irradiated allogeneic (Laz 388) cells was also utilized to stimulate the proliferation of patients' PBMCs obtained at various intervals post-ABMT. The majority of patients responded to TCGF-CM both before and within the first four weeks after transplantation. Although only two of seven patients' cells responded to TCGF-CM in the second month posttransplant, the majority (70%) of patients again were responsive at 12 to 116 weeks. For allogeneic mixed-lymphocyte reactions (MLR), EBV-

Table 4. Serum Immunoglobulin Levels

Immunoglobulin Isotype	No. Patients Normal*/No. Patients Examined										
	Weeks Posttransplant										
	Pre	4	8	12	16	20	24	28-36	40-52	56-90	
IgG	8/13	7/10	4/13	2/7	4/11	2/6	3/6	5/8	2/4	3/4	
IgA	9/13	7/10	3/13	0/7	5/11	2/6	3/6	4/8	2/4	4/4	
IgM	6/13	6/10	3/13	2/7	3/11	2/6	3/6	5/8	3/4	3/4	

*Normal serum levels of IgG, IgA, and IgM were 800 to 1800 mg/dL, 90 to 450 mg/dL, and 60 to 280 mg/dL respectively.

Table 5. T Cell Proliferation

Stimulus	No. Patients Responding*/No. Patients Tested			
	Weeks Posttransplant			
	Pre	0-4	4-8	12-116
Phytohemagglutinin 1:5 or 1:10	3/8	1/9	0/7	4/10
Concanavalin A 1:20 or 1:40	4/8	1/9	0/7	4/10
Mumps 1:20	4/8	2/9	0/7	2/10
Tetanus Toxoid 1:10	4/8	4/9	0/7	2/10
<i>Candida albicans</i> 1:50	4/8	4/9	1/7	2/10
TCGF-CM†	6/8	6/9	2/7	7/10
Laz 388‡	6/8	7/9	4/7	8/10

*The increment in ^3H thymidine uptake by cells cultured with stimulus compared to cells cultured in media alone was calculated. A patient was judged to have demonstrated a response if the increment was $\geq 40\%$ of the increase in proliferation noted in cells obtained from a normal donor cultured with the same stimulus under identical conditions.

†T cell growth factor-containing medium was obtained by stimulating unfractionated peripheral blood mononuclear cells with phytohemagglutinin, phorbol myristate acetate, and irradiated allogeneic (Laz 388) cells.

‡Epstein-Barr virus (EBV)-transformed Laz 388 cells were irradiated and cultured with equal numbers of patient peripheral blood mononuclear cells.

transformed Laz 388 cells were irradiated and cultured with equal numbers of patients' PBMCs. As was true for cultures with TCGF-CM, the majority of patients' PBMCs examined before and in the first month after transplant proliferated in MLR. Responsiveness of PBMCs to allogeneic Laz 388 cells was present in 57% and 80% of patients tested at four to eight weeks and at 12 weeks posttransplant respectively.

We next examined whether proliferation of cells to the combination of TCGF-CM + PHA was enhanced (≥ 1.5 -fold) relative to that noted to either TCGF-CM or PHA alone (Table 6). The majority ($\geq 88\%$) of patients tested both before and at ≥ 12 weeks post-ABMT demonstrated prolifer-

Table 6. T Cell Proliferation*

Patient Response	No. Patients Responding/No. Patients Tested			
	Weeks Posttransplant			
	Pre	0-4	4-8	12-116
TCGF + PHA ≥ 1.5	7/8	3/9	4/7	9/10
TCGF + PHA ≥ 1.5	8/8	3/9	4/7	3/10
Laz 388 + TCGF ≥ 1.5	7/8	4/9	6/7	9/10
Laz 388 + TCGF ≥ 1.5	7/8	7/9	4/7	7/10
Laz 388	8/8	9/9	7/7	10/10

*20 μL of T cell growth factor containing media (TCGF-CM) was added to wells already containing patient cells and/or media, phytohemagglutinin (PHA), or Laz 388 cells. The addition of TCGF-CM to PHA or Laz 388 was judged to significantly enhance proliferation if it increased proliferation by 50% compared to the proliferation noted to PHA or Laz 388 alone.

ation to TCGF-CM + PHA: proliferation to PHA ratio ≥ 1.5 . In contrast, within the first two months posttransplant $\leq 50\%$ of patients manifested a greater response to TCGF-CM + PHA than to PHA alone. All eight patients examined prior to transplant had significantly increased proliferation to TCGF-CM + PHA relative to TCGF-CM alone. However, the addition of PHA to TCGF-CM did not significantly increase proliferation relative to that noted to TCGF-CM alone in the majority of patients examined up to 116 weeks posttransplant.

In a similar fashion we tested whether patients' PBMCs proliferated to a greater extent to TCGF-CM + allogeneic (Laz 388) cells than to either of these stimuli alone. Nearly all patients tested before and at ≥ 12 weeks posttransplant (seven of eight and nine of ten patients respectively) demonstrated increased proliferation to Laz 388 cells + TCGF-CM compared to TCGF-CM alone. Similarly, most ($\geq 70\%$) patients examined before and at ≥ 12 weeks posttransplant augmented proliferation to Laz 388 cells + TCGF-CM compared to Laz 388 cells alone. At one and two months posttransplant, 44% to 77% of patients also proliferated to a greater extent to the combination of TCGF-CM + Laz 388 cells than to either stimulus alone.

Natural killer cell function. Cytotoxicity assays were performed using effector cell (patient's PBMCs): target cells (K562 chronic myelogenous leukemia) ratios of 7.5 to 60:1. Significant killing was evidenced by $>5\%$ of maximal $^{51}\text{chromium}$ release from radiolabeled target cells. As can be seen in Table 7, all six patients tested prior to transplantation demonstrated natural killer (NK) cell function. Early (zero to four weeks) after transplant cytotoxicity was noted in seven of 11 patients tested. All patients tested at four to eight weeks and ≥ 12 weeks (seven and nine patients respectively) post-ABMT had normal NK cell function. In each case maximal killing was seen at effector: target ratios of 60:1.

Natural killer cell killing against autologous tumor cells was examined in a single patient at one-month post-ABMT. Twenty-two percent of the patient's PBMCs were NK cells by virtue of staining with anti-NKH1 MoAb at this time. Cryopreserved tumor cells were radiolabeled with $^{51}\text{chromium}$, and 19% maximal $^{51}\text{chromium}$ release was noted at patient's PBMCs (effector):cryopreserved autologous tumor cell (target) ratios of 20 to 40:1.

DISCUSSION

In the present report we have examined both hematologic engraftment and immunologic reconstitution in recipients of autologous bone marrow purged with anti-B1 MoAb and complement. Hematologic engraftment was prompt with return of $\geq 0.5 \times 10^3/\mu\text{L}$ granulocytes and $\geq 2 \times 10^4/\mu\text{L}$ platelets at a median of 26 and 29 days posttransplant, respectively. Virtually all blood product support was complete by eight weeks posttransplant. In contrast to hematologic engraftment, immunologic reconstitution was prolonged. As expected, anti-B1 and complement marrow treatment completely eradicated detectable B cells as well as totally abrogating normal B cell function in vitro and in vivo. Normal numbers of circulating B cells consistently returned by five months post-ABMT, whereas return of immunoglo-

Table 7. Natural Killer Cell Function

No. Patients Demonstrating Cytotoxicity*/No. Patients Tested				
Weeks Posttransplant				
Pre	0-4	4-8	12-116	
6	7	7	9	
6	11	7	9	

*Assays were performed at effector:target ratios of 7.5 to 60:1 using 5,000 K562 target cells/well. Greater than 5% maximal chromium release was judged as significant killing of target cells.

bulins in some patients did not occur for one year. Concomitant with the appearance of normal numbers of circulating B cells was the return of in vitro responses of B cells to triggers of activation (anti-u). Although normal numbers of T cells were evident within the first several months post-ABMT, a reversed T4:T8 ratio persisted in some patients indefinitely (up to three years). Moreover, in vitro responses to T cell mitogens and specific antigens were again not normalized for at least three months. Phenotypically and functionally defined NK cells appear to have a relative growth advantage early after transplant. These studies demonstrate that anti-B1 purged autologous bone marrow can result in complete hematologic and immunologic engraftment, although T and B cell engraftment appears to be prolonged. Most importantly, the time to hematologic and immunologic engraftment has not been associated with any significant acute or chronic clinical toxicities.

Overt or occult bone marrow involvement is very common at the time of relapse of NHL, regardless of the initial histology,²²⁻²⁶ and it therefore seemed important to develop methods to eradicate tumor cells from the marrow. We have attempted to purge tumor cells from the marrow using the pan-B cell MoAb anti-B1 and complement. To date, all patients have responded to the ablative therapy with a complete response and no acute toxicity, all have engrafted hematologically, and all were discharged from the hospital. Our patients achieved $\geq 0.5 \times 10^3$ granulocytes/ μL at a median of 26 (range 12 to 45) days post-ABMT, which is not significantly prolonged when compared to previous reports for nonpurged or pharmacologically purged autologous marrow.^{8-11,14,18} These results are slightly delayed when compared to recipients of syngeneic² or allogeneic⁶ marrow grafts, but it is important to note that autologous marrow in most instances had seen significant prior cytotoxic therapy. Moreover, although most patients developed fever during neutropenic periods, no increased numbers of bacterial ($n = 2$) or fungal ($n = 1$) septic infections were noted in our series compared to previously reported trials. Stable nontransfused platelet counts of at least $2 \times 10^4/\mu\text{L}$ were attained at a median of 29 (range 14 to 137) days in our series compared to recipients of syngeneic marrow who no longer required platelets at a median of 16 (range nine to 45) days² or to recipients of allogeneic and autologous transplants who achieved sustained untransfused platelet counts of $5 \times 10^4/\mu\text{L}$ at a median of 20 to 34 days respectively.^{8-11,14,18} In our series, 80% of platelets were transfused in the first month, 19.5% in the second month, and only 0.5% beyond eight weeks. There was not a single episode of clinically

significant bleeding. These results suggest that hematologic engraftment following anti-B1 purging is delayed but has not led to significant acute clinical toxicities.

The question of whether anti-B1 purging will become a more generalized treatment modality for patients with NHL will depend on the answers to two questions: first, and most important, is whether this purging technique can effectively deplete the clonogenic tumor cell; and second is whether the marrow purging affects engraftment sufficiently to produce clinical toxicity. The first question is still unanswered. Our results suggest that purging does not appear to produce significant clinical toxicity, which is surprising considering (1) the time to B and T cell engraftment observed in this study; and (2) the fact that many of our patients had abnormal numbers of B and T cells, NK cells, and T4:T8 ratio pretransplant attributable to their underlying disease and its chemotherapeutic treatment⁴⁶ as well as to multiple transfusions.⁴⁷ Less than 50% of recipients of anti-B1 purged autologous bone marrow have normal numbers of B cells either pretransplant or within the first two months post-ABMT, but all patients examined at ≥ 20 weeks post-ABMT have achieved normal numbers of phenotypically identified B cells. Fifty percent and 100% of our patients examined at ≥ 6 months and one year post-ABMT, respectively, achieved normal serum Ig levels, confirming the return of *in vivo* B cell function. Moreover, *in vitro* examination of B cells at ≥ 12 weeks post anti-B1 marrow purging also confirmed normal B cell responses to triggers of activation. Since the B1 antigen is expressed on 50% of pre-B cells in the marrow⁴⁸ and on virtually all mature B cells excluding the plasma cell,²⁸⁻³⁰ the delayed time to B cell engraftment in our patients suggests that many pre-B cells and most mature B cells have been eradicated by the *in vitro* purging and the *in vivo* ablative therapy. The present data do not permit us to determine whether the time to B cell engraftment is simply due to repopulation of B cell progenitors or whether it is also due to the defects in T cell reconstitution. Future studies of B cell reconstitution in the bone marrow may provide insight into this question.

Although return to normal B cell function *in vivo* requires approximately six months, it is important to note that infections associated with agammaglobulinemia have not been seen post-ABMT. B cell phenotype and function have been examined postallogeneic transplantation therapy for leukemia⁴⁹⁻⁵⁹; however, there have been few studies detailing B cell reconstitution postsyngeneic⁶⁰ or post-ABMT for leukemia^{54,56,61} and no similar studies post-ABMT for lymphoma. It is therefore difficult to determine whether B cell engraftment in our hands is significantly prolonged. Moreover, it is not possible to compare B cell engraftment post-ABMT with that noted after allogeneic transplant because of the immune dysregulation of graft-v-host disease (GVHD) and prolonged immune suppression by drugs in the majority of allogeneic studies as well as the immune defects following T cell depletion in more recent studies in recipients of allogeneic transplants. We have studied one patient who underwent an identical twin transplant for relapsed B cell lymphoma. In this case B cells returned between six and eight weeks and normal Ig levels were present by four months. This suggests that anti-B1 purging does prolong

engraftment, although it is important to note that the autologous bone marrow had seen significant prior cytotoxic therapy. Further studies of B cell reconstitution post-ABMT in patients who receive nonpurged marrows will be necessary to determine whether anti-B1 MoAb and complement treatment significantly impairs B cell function.

Whereas B cell immunosuppression did not result in clinical toxicity, T cell immunosuppression appears to result in increased episodes of Herpes Zoster. In our series approximately one third of patients who are disease free at ≥ 6 months have developed Zoster without a significant increase in other viral or fungal infections. Other investigators have noted interstitial pneumonitis and cytomegalovirus (CMV) infections in recipients of nonpurged autologous grafts that we have not observed to date.⁶² These specific immune defects in our patients are thus far unresolved. Although normal numbers of T cells were observed by two months post-ABMT, reversed T4:T8 ratio persists for most patients past one year. These phenotypic abnormalities correlated with abnormal T cell responses to mitogens and antigens *in vitro*. Responsiveness of T cells to mitogens was largely absent in all patients within the first two months and was present in $< 50\%$ at 12 to 116 weeks post-ABMT, reflecting a relative decrease in T4⁺ cells. In contrast, responsiveness to either TCGF-CM or allogeneic cells was noted in the majority of patients at ≥ 12 weeks post-ABMT. It is interesting to note that the addition of TCGF-CM to cells stimulated with PHA-LCM or allogeneic cells led to greater proliferation than the proliferation induced by either stimulus alone. This suggests that there may be a relative excess of activated T cells expressing TCGF receptors but that there may be a deficiency of interleukin 2 (IL 2) post-ABMT. A relative deficiency of IL 2 has been previously reported early following allogeneic transplant.⁶³⁻⁶⁵ Since the provision of external IL 2 containing media led to augmentation of T cell proliferation, these T8⁺ cells appear to respond in a normal fashion to TCGF. Again these T cell defects observed *in vitro* do not convert into significant clinical toxicities.

It is of great interest to note that 10% to 15% of PBMCs during the first three weeks post-ABMT express the NKH1 antigen. These cells have both the phenotypic and functional characteristics of NK cells. Large numbers of these cells ($\geq 10\%$ marrow PBMCs) are infused in the graft and may have a selective growth advantage early following ABMT. Previous studies have demonstrated that several NKH1 positive clones isolated from a patient post-allogeneic transplant had demonstrated marked NK activity not only against K562 but also against cryopreserved autologous tumor cells.⁶⁶ The demonstration of *in vitro* cytotoxicity of these NK cells noted post-B1 purging against cryopreserved tumor cells further suggests that these cells may have an *in vivo* role in killing small numbers of residual tumor cells within the host. Depletion of this antitumor effect of NK cells post-ABMT coupled with definition of the factors regulating growth of these cells may offer important insights into the treatment of human tumors.

Our studies suggest that purging autologous bone marrow with anti-B1 MoAb and complement does not inhibit hematologic and immunologic engraftment post-ABMT. More importantly, minimal acute and chronic clinical toxicities are

observed following this treatment modality. The next step in these studies is to improve in vitro ablation by first using multiple antibodies and then comparing complement-mediated lysis to other forms of in vitro ablation. With each of these future studies we must carefully examine not only the in vitro tumor ablation but also the effects of hematologic and immunologic engraftment. Moreover, it is hoped that a better understanding of the growth and regulation of B and T cells will permit us to modulate their growth in vivo and thereby to hasten engraftment.

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Association Between Gelatinase Release and Increased Plasma Membrane Expression of the Mo1 Glycoprotein

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Mo1, a glycoprotein heterodimer (gp 155,95) that functions as an adhesion promoting molecule and as the C3bi receptor of human myeloid cells, is expressed in increased amounts in the plasma membrane after exposure of polymorphonuclear leukocytes (PMNs) to various stimuli. Previous studies have suggested that secondary granules represent an intracellular pool of Mo1 that, upon degranulation, fuse with the plasma membrane resulting in a tenfold increase in surface expression of Mo1. To determine the intracellular location of Mo1, we monitored Mo1 expression by immunofluorescence and compared it to the release of myeloperoxidase (MPO, a marker for the primary granules), vitamin B₁₂ binding protein (B₁₂BP, secondary granules), and gelatinase (gelatinase-containing organelles) following exposure to various stimuli. Human neutrophils stimulated with 20 mmol/L fluoride for 16

minutes exhibited a twofold increase in Mo1 expression and gelatinase release but no enhanced release of primary or secondary granular contents. In a similar fashion, incubation of cells at 37° C for five minutes with 7.5×10^{-9} to 10^{-6} mol/L N-formyl-methionyl-leucyl-phenylalanine (FMLP) resulted in significant increases in both surface Mo1 expression (three- to fivefold) and gelatinase release (five- to eightfold) without significant release of either MPO or B₁₂BP. In addition, both the fluoride and FMLP experiments demonstrated that Mo1 up-modulation alone is not sufficient to activate superoxide (O₂⁻) production. These data indicate that at least one intracellular storage pool of Mo1 is the gelatinase-containing organelles and that their fusion with the plasma membrane results in increased expression of Mo1 on the cell surface.

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THE SURFACE of human myeloid cells contains a glycoprotein, Mo1, a heterodimeric glycoprotein with subunit molecular weights of 155 kD and 94 kD.¹ Mo1, which is equivalent to glycoproteins termed elsewhere as OKM1² and Mac-1,³ serves as the receptor for C3bi-opsonized particles.⁴⁻⁶ In addition, the Mo1 glycoprotein also plays a role in various aspects of cellular adhesion such as adherence, spreading, chemotaxis,⁷ and PMN aggregation.⁸ Upon exposure to various degranulating stimuli, the surface expression of Mo1 increases by up to tenfold, as determined by immunofluorescence,⁹⁻¹¹ enhanced C3bi receptor activity,^{8,12} and PMN aggregation.⁸ This up-regulation of Mo1 appears to be due to the translocation of a large intracellular pool of the glycoprotein to the cell surface.⁹⁻¹³ Evidence for this intracellular location is twofold. First, permeabilized PMNs stained with a fluorescent monoclonal antibody (MoAb) directed against Mo1 show a diffuse granular fluorescence pattern.¹³ Second, subcellular fractionation studies show Mo1 to be present in the fraction enriched for secondary granules as well as in the membrane fraction.^{9,10,13} On the basis of these data it has been postulated that degranulation in PMNs results in the translocation of the secondary granule-associated Mo1 to the plasma membrane, where it is then detected in increased amounts.⁹

While the above data support a secondary granule location for the intracellular pool of Mo1, several lines of experimental evidence suggest that this glycoprotein might be contained in yet another intracellular compartment. First, the surface expression of Mo1 increases under conditions in which primary and secondary granules are not released, such as incubating cells in suspension at 37° C.^{10,13,14} Second, a population of "granules" has recently been described, the gelatinase-containing organelles, that do exhibit this type of lability when incubated at 37° C.¹⁵ Using gelatinase release to monitor these organelles, Dewald et al demonstrated that the gelatinase-containing organelles fuse with the membrane during warming of PMNs as well as during exposure to low (chemotactic) concentrations of FMLP.¹⁵ These experimental conditions caused little, if any, release of primary or secondary granule contents.^{14,15} Third, secondary granules

and gelatinase-containing organelles have nearly identical densities and are therefore difficult to resolve from each other except under fractionation conditions where sedimentation velocity is used.¹⁵ Under the conditions used in the previous reports of Mo1 subcellular localization, the secondary granule fraction was probably contaminated with gelatinase-containing organelles, since the fractionation technique involved density separation.^{9,10} Taken together, these observations raise the possibility that Mo1 may reside in the gelatinase-containing organelles.

To test this possibility, Mo1 surface expression under a variety of conditions was compared to the extracellular release of the contents of primary and secondary granules as well as the gelatinase-containing organelles. Two stimuli were employed, fluoride and FMLP. Fluoride has been previously shown to stimulate the respiratory burst in neutrophils without the concomitant release of markers of either primary granules (myeloperoxidase, β -glucuronidase, lysozyme) or secondary granules (lysozyme, vitamin B₁₂ binding

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protein [$B_{12}BP$]).^{16,17} FMLP at low concentrations causes substantial release of gelatinase with minimal release of primary and secondary granule contents.^{15,18} The results reported here indicate that both of these stimuli cause substantial MoI expression and that this is correlated with release of gelatinase but not markers for the primary and secondary granules.

MATERIALS AND METHODS

Chemicals. Sodium fluoride, sodium chloride, and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific, Fairlawn, NJ. ³H-acetic anhydride and (¹⁴C)-vitamin B_{12} (90 cpm/pg) were purchased from Amersham, Arlington Heights, IL. Ionophore A23187 (Calbiochem, LaJolla, CA), FMLP (Calbiochem), and cytochalasin B (Sigma, St. Louis) were dissolved in DMSO and then added to aqueous solutions with vigorous mixing. All other reagents were purchased from Sigma, St. Louis, unless otherwise stated.

Monoclonal antibodies. Cell surface antigens were monitored by indirect immunofluorescence analysis using monoclonal antibodies (MoAbs) from mouse ascites specific for MoI (IgG-2a subclass)⁵; β -2 microglobulin (IgG-2b subclass), a gift of Dr Lee Nadler, Boston¹⁹; and Ia (IgG-2a subclass).²⁰

Cell preparation. Suspensions of PMNs were prepared from acid citrate dextrose-treated blood by a previously reported procedure.²¹ Except for the step of sedimentation in dextran that was performed at room temperature, cells were kept at 4° C throughout the preparation.

Cell stimulation. For FMLP and A23187 stimulation, cells were diluted to 2×10^6 cells/mL in phosphate buffered saline (PBS)²² (138 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4 , 1.5 mmol/L KH_2PO_4 , 0.9 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, and 7.2 mmol/L glucose, pH 7.4) and prewarmed to 37° C. Cells were incubated for five minutes at 37° C with either FMLP (concentrations ranging from 10^{-6} to 10^{-9} mol/L) or A23187 (10^{-6} mol/L). Cytochalasin B (10 μ mol/L) was added to some control reaction mixtures to induce maximal degranulation. The total concentration of DMSO (the solvent for cytochalasin B and FMLP) never exceeded 2.5 μ L/mL and had no effect on O_2^- production. After incubation, reaction mixtures were transferred to an ice bath and then centrifuged at 600 g for five minutes at 4° C. The cells in the pellets were used for MoI analysis and for determination of total enzyme content, while the supernates were used for measurements of enzyme release. For fluoride stimulation, a modified PBS (PBS') was used so that the final salt concentrations would be physiologic after 20 mmol/L NaF (final concentration) was added.¹⁶ The nonstimulated cells in PBS' received 20 mmol/L NaCl to restore isotonicity. During fluoride stimulation, cells were incubated for up to 17 minutes at 37° C with the samples taken at various times and treated as described above for FMLP stimulated cells.

Measurements of O_2^- production. Superoxide dismutase-inhibitable cytochrome *c* reduction was used to measure O_2^- as previously reported²³ except that the cytochrome *c* concentration was 150 μ mol/L. Cells were prewarmed to 37° C for two minutes prior to stimulation and were used at a final concentration of 2×10^6 cells/mL in a final volume of 1.9 mL. Cytochrome *c* was not limiting as the maximum amount of O_2^- produced was 100.0 ± 6.5 nmol (mean $\pm$ SD), which was less than the 150 nmol available for reduction in the reaction mixture.

Immunofluorescent analysis of membrane antigens. Indirect immunofluorescence of MoI and β -2 microglobulin was measured by flow cytometry as described by Todd et al.⁹ The channel number (log scale) representing the mean fluorescence intensity of 5,000 cells, which had been exposed to either an experimental or a negative control antibody (anti-Ia) and then to a fluorescein-conjugated goat

antimouse immunoglobulin, was determined. Logarithmic channel numbers were converted to linear values,²⁴ and the specific mean fluorescence intensity for cells stained by experimental antibodies was calculated by subtracting the mean channel number of cells exposed to the negative control antibody from that of cells stained by the experimental antibodies. To compare data from one experiment to another, the specific mean fluorescence of experimentally treated cells was compared in each experiment to that resulting from stimulation of neutrophils with 10^{-6} mol/L A23187 in the presence of 10 μ mol/L cytochalasin B for five minutes at 37° C, a value that was defined as the maximum MoI expression.

Granule marker analysis. $B_{12}BP$ was measured by a previously reported modification¹⁴ of the method of Gottlieb et al.²⁵ MPO was measured by the method of Bretz and Baggiolini.²⁶ Lactic dehydrogenase (LDH) was measured as previously described²⁷ and was used as a measure of cellular integrity during stimulation. The extent of release of each of these markers was expressed as the percent of total cellular activity present in the cell supernate following stimulation.

Gelatinase activity was measured by the release of tritium counts from gelatin labeled with ³H-acetic anhydride by the method of Dewald et al.¹⁵ Before samples were assayed they were treated with 3 mmol/L di-isopropylphosphorofluoridate (DFP) to inhibit serine proteases.¹⁵ Gelatinase-dependent tritium release was defined as the difference between the total counts released by the extracellular medium samples and the counts released in the presence of buffer alone. Gelatin breakdown was found to be linear with substrate concentration from 20 to 100 μ g/mL, time from 1.5 to 3.0 hours, and cell concentration from 5×10^5 to 5×10^6 cells/mL. In a typical assay containing substrate at a concentration of 20 μ g/mL (total 42,000 cpm) and supernates from cell suspensions (2×10^6 cells/mL) incubated under various conditions, the following counts were released from gelatin: unstimulated cells at 4° C for five minutes, 90 cpm/ 10^5 cells; 37° C for five minutes without stimulus, 360 cpm/ 10^5 cells; and 37° C for five minutes with 10^{-6} mol/L A23187 and 10 μ mol/L cytochalasin B, 1030 cpm/ 10^5 cells. The presence of an inhibitor in the cytosol resulted in an inability to measure accurately the total activity in the intact cell.²⁸ Therefore, gelatinase release was expressed not as percent release as with the other markers but instead as the absolute amount of substrate degraded by the extracellular fluid derived from the incubation of a fixed number of cells. Maximum release was defined as the gelatinase activity present in the extracellular medium following stimulation of neutrophils with 10^{-6} mol/L A23187 and 10 μ mol/L cytochalasin B for five minutes.

RESULTS

In Fig 1 the effects of temperature and fluoride on MoI surface expression and gelatinase release are shown. The data are expressed as the percent of maximum response defined as that observed in the presence of A23187 and cytochalasin B. It can be seen that warming PMNs from 4° C to 37° C resulted in a significant increase in both gelatinase release (1.8-fold) and surface MoI expression (1.8-fold) without concomitant release of MPO or $B_{12}BP$, markers for the primary and secondary granules, respectively. Subsequent stimulation of the prewarmed PMNs by fluoride caused a further increase in gelatinase release (1.85-fold) and in MoI expression (2.0-fold) above the 37° C control, again without release of primary or secondary granules. Control experiments showed no release of LDH (data not shown), indicating cellular integrity. In addition, $B_{12}BP$ and MPO could readily be detected by our assay techniques when cells were exposed to A23187 and cytochal-

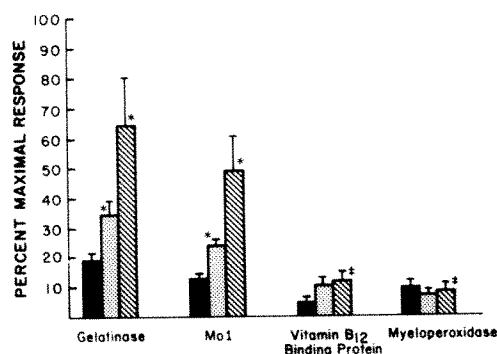


Fig 1. Fluoride stimulation of Mo1 expression and granule marker release. PMNs were incubated in 20 mmol/L fluoride and PBS' for 15 minutes (▨) before Mo1 expression and enzyme release were measured. NaCl was added to unstimulated cells at 4° C (■) and at 37° C (▩) to bring the PBS' to isotonicity. The brackets indicate the SEM for six experiments. The maximum response was defined as the amount of activity measured after cells were incubated with 10^{-6} mol/L A23187 and 10 μ mol/L cytochalasin B for five minutes. Average maximum values ($\pm$ SEM) are as follows: gelatinase, 1028 ± 295 cpm/ 10^6 cells; Mo1, 130 ± 29 mean fluorescence units/cell (arbitrary units); B₁₂BP release, $39\% \pm 9.2\%$ of total cell B₁₂BP; and MPO release, $53.5\% \pm 4.5\%$ of total cell MPO. * $P < 0.006$ by paired t test for 4° C v 37° C and 37° C v F1-. ‡Difference is not significant for 37° C v F1-.

asin B, indicating that failure to correlate fluoride-induced Mo1 expression with granule release was not due to adherence of the granule proteins to the cell surface. The levels of MPO and B₁₂BP released were substantial and not statistically different from each other.

Experiments were then performed to determine the time courses of Mo1 expression and gelatinase release following fluoride stimulation. In addition, O₂⁻ production was measured simultaneously to see if its activation was correlated temporally with either gelatinase release or Mo1 up-modulation. As shown in Fig 2, both Mo1 surface expression and gelatinase release rise concomitantly, beginning as early as three minutes after the addition of fluoride and continuing through the entire 16-minute incubation. In contrast, there was no significant release of B₁₂BP and MPO under these conditions. Superoxide production did not correlate with either Mo1 expression or gelatinase release, as it began substantially later after its characteristic nine-minute lag period.^{16,17} Thus the appearance of Mo1 on the cell surface and the release of gelatinase were closely correlated in time and appeared to occur independently of O₂⁻ production and the release of primary and secondary granules.

Since Dewald et al have previously reported that chemotactic concentrations of FMLP (10^{-9} to 10^{-8} mol/L) cause significant gelatinase release with minimal release of B₁₂BP and MPO,¹⁵ the effect of FMLP on Mo1 surface expression was next determined. Figure 3 shows the effect of various concentrations of FMLP on the expression of Mo1 and the release of gelatinase. Increases in cell surface Mo1 and gelatinase release occurred in parallel at all concentrations of the stimulus except at the lowest concentration of FMLP where the level of sensitivity of the gelatinase assay was limiting and little or no gelatinase activity could be detected.

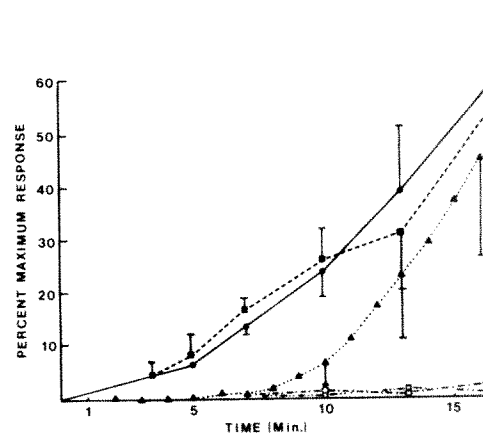


Fig 2. Time course of Mo1 expression, superoxide production, and release of gelatinase, B₁₂BP, and MPO following fluoride stimulation. Mo1 expression (■), gelatinase release (●), O₂⁻ production (▲), B₁₂BP release (○), and MPO release (□), were measured at various times after fluoride (20 mmol/L) had been added to 2×10^6 PMNs/mL at 0 minutes. The data from four experiments are expressed as percentage of maximum response (cells exposed to 10^{-6} mol/L A23187 and 10 μ mol/L cytochalasin B for five minutes) $\pm$ SEM. The values for the unstimulated control did not change during the course of the incubation and were subtracted from the fluoride measurements. The maximum responses ($\pm$ SEM) were as follows: for Mo1, 98 ± 43 mean fluorescence units/cell; for gelatinase release, $1,035 \pm 316$ cpm/ 10^6 cells; for B₁₂BP release $47\% \pm 6\%$ of total cell activity; for MPO release $48\% \pm 9\%$ of total cell activity; and for O₂⁻ production, 108 ± 10 nmol/min/ 1×10^7 cells (initial velocity).

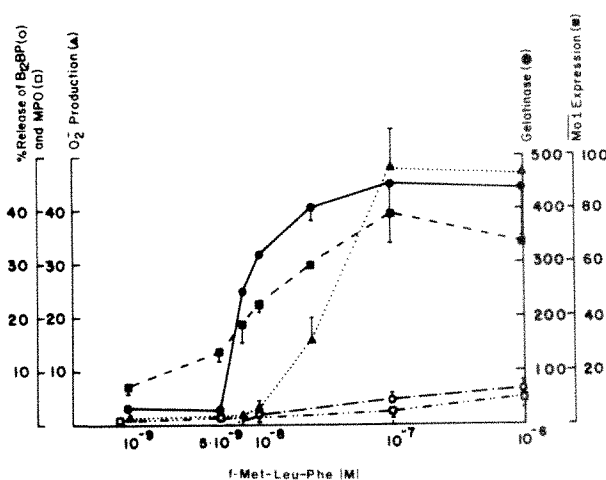


Fig 3. The effect of FMLP concentration on Mo1 expression, O₂⁻ production, and release of gelatinase, B₁₂BP, and MPO. Separate aliquots of PMNs at 2×10^6 cells/mL were exposed to different concentrations of FMLP (10^{-9} to 10^{-8} mol/L) for five minutes at 37° C and assayed for Mo1 expression, O₂⁻ production, and enzyme release as described in "Methods." Activity in unstimulated cells incubated at 37° C for five minutes was subtracted from the values obtained with stimulated cells. The data from four experiments are expressed as the mean $\pm$ SEM for: Mo1 expression (■), in arbitrary mean fluorescence units; gelatinase release (●), in cpm/ 10^6 cells; O₂⁻ production (▲), in nmol/min/ 10^7 cells; and for granule marker release [B₁₂BP (○) and MPO (□)] in percentage of total cell activity.

At 10^{-8} mol/L FMLP, Mol expression and gelatinase release were nearly maximal, while there was insignificant release of both B_{12} BP and MPO. Only at higher concentrations of FMLP (10^{-7} mol/L) was the release of primary or secondary granule contents observed. In all cases minimal LDH was released (data not shown). As with MPO and B_{12} BP release, higher concentrations of FMLP (greater than 2.5×10^{-8} mol/L) were required to activate O_2^- production. Thus increases in the expression of Mol on the cell surface and the release of gelatinase were closely correlated at all concentrations of FMLP and could be dissociated from O_2^- production and from release of primary and secondary granules.

DISCUSSION

Our data suggest that human PMNs exhibit at least three levels of activation with respect to Mol expression and exocytosis. Upon intense stimulation (eg, with A23187 and cytochalasin B), substantial amounts of Mol are expressed on the cell surface while secondary granules, primary granules, and gelatinase-containing organelles all release their contents into the extracellular medium. At the other extreme, in which the cells are in their basal or unstimulated state (defined experimentally as PMNs maintained at 4°C), only 10% as much Mol is expressed and little, if any, exocytosis occurs. An intermediate state of activation can also be identified, perhaps part of a continuum of the activation process, in which approximately 50% of the maximal Mol is expressed on the cell surface and in which only the gelatinase-containing organelles undergo appreciable exocytosis. Stimuli such as fluoride and FMLP (at low concentrations) cause this intermediate level of activation and provide an experimental system for correlating Mol expression with exocytosis of gelatinase-containing organelles.

We provide here strong evidence that in the basal and intermediate levels of activation, Mol expression is not related to the release of primary or secondary granules. When maintained at 4°C , neutrophils express Mol without any ongoing exocytosis, indicating that there is a certain amount of Mol already present in the plasma membrane. With fluoride as a stimulus, Mol expression increases to approximately 50% of its maximum without any net increase in the release of B_{12} BP or MPO (Figs 1 and 2). These data are consistent with previous reports demonstrating absence of primary and secondary granule release in response to fluoride.^{16,17} Similar results were obtained with FMLP at concentrations below 10^{-8} mol/L where more than 50% of the Mol is expressed with less than 10% release of the primary and secondary granules.¹⁵ Taken together these data indicate that the primary and secondary granules do not contain the intracellular reserves of Mol that are expressed in the intermediate state(s) of PMN activation.

If this labile reserve pool of Mol is not located in the primary and secondary granules, where is it located? One possibility would be cryptic sites within the plasma membrane that are exposed following stimulation. This, however, does not appear to be the case based on two findings in

previous reports.^{9,13} The first observation was that the Mol antigen was found to be located predominantly in a secondary granule-containing fraction and present only in small amounts in the plasma membrane fraction.^{9,13} The second was that immunofluorescent staining of PMNs with the Mol antibody showed a granular intracellular staining pattern.¹³ Based on these studies it would appear that the Mol reserve pool is located within the cell and not in the membrane. Two observations in the literature suggest that the gelatinase-containing organelles may be one major intracellular site. First, gelatinase-containing organelles have a density nearly identical to that of the secondary granules and would therefore co-purify on density gradients with the secondary granule fraction that reacts with Mol.^{9,10,13,15} Second, the gelatinase-containing organelles are quite labile and readily fuse with the plasma membrane upon warming or gentle stimulation, properties that are strikingly similar to the Mol expression seen in the intermediate stages of activation. We now provide data showing a strong correlation between Mol expression and the fusion of gelatinase-containing organelles with the plasma membrane. With fluoride, the time course of Mol expression and gelatinase release closely parallel each other, while in the FMLP experiments, the dose-response curves for Mol and gelatinase are strikingly similar. In quantitative terms, fluoride causes the release of 60% of the releasable gelatinase, a value that agrees closely with the 50% maximal Mol expression induced by this stimulus. A similar quantitative relationship is observed with FMLP. Thus the intermediate level of Mol expression occurs in the absence of primary and secondary granule release and strongly correlates with exocytosis of the gelatinase-containing organelles.

Since approximately 50% of the maximal Mol is expressed under conditions where roughly 50% of the maximal gelatinase is released, it is tempting to speculate that the remaining Mol is also located in the gelatinase-containing organelles. Because of the lack of specificity of the gelatinase assay, however, the possibility that some Mol is located in the secondary granules cannot be excluded. The substrate employed in the gelatinase assay (derived from type I collagen) can be degraded by neutrophil collagenase²⁹ present in the secondary granules³⁰ as well as by elastase in the primary granules.³¹ Experimental conditions which result in maximum Mol expression (A23187 + cytochalasin B) also cause the release of substantial amounts of primary and secondary granule contents.¹² The collagenase and elastase thus released contribute to the total gelatinolytic activity and make it difficult to determine the contribution of the gelatinase-containing organelles. Because of these technical limitations, the most that can be concluded from the data is that at least 50% of the Mol that is eventually expressed is contained in the gelatinase-containing organelles. Whether the remaining 50% is localized to the same organelles or to the specific granules can only be determined by direct measurements of Mol in subcellular fractions in which specific granules are resolved from the gelatinase-containing organelles. This, however, requires the sophisticated technology of rate zonal sedimentation.

The possibility that at least some of the intracellular pool

of Mo1 is contained in the specific granules is supported by studies on a patient with specific granule deficiency.¹³ Mo1 expression in these experiments was determined by measuring the number of C3bi receptors on the cell surface. PMNs from the patient exhibited abnormal up-regulation of C3bi in response to stimulation, suggesting a specific granule location for at least part of the Mo1. Whether the gelatinase-containing organelles in this patient were also abnormal, however, was not reported.

Mo1 expression does not appear to be sufficient to activate O_2^- generation. In both the fluoride and FMLP experiments, substantial amounts of Mo1 were expressed without concomitant O_2^- generation. We cannot exclude the possibility, however, that Mo1 might still be necessary for respiratory burst activation, since we have not observed O_2^- production in the absence of Mo1 up-regulation.

The localization of at least 50% of the intracellular Mo1 in the labile gelatinase-containing organelles may have physio-

logic relevance. During chemotaxis when PMNs are exposed to low concentrations of chemotactic agents like FMLP, gelatinase-containing organelles will fuse with the plasma membrane and cause increases in both the amount of Mo1 expressed on the cell surface and the amount of gelatinase in the extracellular medium. This increase in Mo1 would enhance PMN attachment to surfaces, a necessary step required for efficient chemotaxis of PMNs through tissues. The gelatinase could serve to partially degrade connective tissue that might impede the mobility of the PMNs. Finally, this early deployment of Mo1 would also eventually facilitate recognition and ingestion of microbes opsonized with C3bi, since it also functions as the C3bi receptor.

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Gene Transfer to Primary Normal and Malignant Human Hemopoietic Progenitors Using Recombinant Retroviruses

By Donna E. Hogge and R. Keith Humphries

To study the feasibility of using retroviruses for gene transfer into human hemopoietic cells, various cell types were exposed to virus carrying the gene for neomycin resistance (*neo'*). In preliminary studies using K562 cells as targets, we found that high viral titer and co-cultivation with viral producer cells rather than incubation in medium exposed to viral producer cells were important variables for achieving high frequencies of G418 resistant (G418^r) colonies. The maximum frequency of G418^r K562 colonies after co-cultivation with cells producing a *neo'* virus titer of 4×10^6 cfu/mL was 60%. When primary human progenitors from normal marrow, fetal liver, or chronic myelogenous leukemia blood were exposed to high titer

viral stocks, both with and without helper virus, under conditions optimized for K562 cells, maximum frequencies of G418^r colonies were 3% to 16% for granulocyte macrophage progenitors and 2% to 6% for primitive erythroid progenitors. The presence of the *neo'* gene in both G418^r K562 and primary hemopoietic colonies was verified by Southern blot. Expression of the *neo'* gene was shown by RNA spot blot. These data demonstrate efficient transfer and expression of the *neo'* gene in both K562 cells and primary human hemopoietic cells from normal and leukemic individuals.

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THE DELIVERY of exogenous genetic material into various target cells is important for many experimental and clinical goals. Recombinant retroviruses provide an attractive vehicle for gene transfer for a number of reasons. These include the high efficiency with which they are able to enter cells and integrate into host DNA, their wide target cell range, and their lack of toxicity.¹ Mammalian bone marrow is a convenient source of primitive cells with high proliferative and self-renewal capacity on which to test the feasibility of using a gene transfer technique to primary cells. A number of workers have already shown that it is possible to introduce a variety of foreign genes into the bone marrow stem cells of mice using recombinant retroviruses.²⁻⁷ These authors have shown a high efficiency of gene transfer, and the transferred gene has remained stably integrated through serial marrow transplants. Although some expression of the transferred genes has been demonstrated, difficulties in obtaining satisfactory levels of the products of certain genes such as human adenosine deaminase have been encountered.⁷ In addition, the long-term stability of gene expression has been questioned.⁵ Nevertheless, it is clear that retroviruses are capable of delivering functional genes into murine hemopoietic progenitors.

Retroviruses have also been used for gene transfer to human cells, although the available data are less extensive than for murine targets.⁷⁻¹² The enzyme deficiency in hypoxanthine phosphoribosyl transferase (HPRT) negative human fibroblasts and B lymphoblasts has been corrected by infection with recombinant virus containing the HPRT gene.^{8,9} Similar results have been reported for experiments in which a virus containing the human adenosine deaminase (ADA) gene was used to infect human ADA-deficient B lymphocytes.⁷ Some data exist for cells of the hemopoietic system from experiments in which long-term human marrow cultures were infected with virus containing oncogenes or the human HPRT gene.^{10,11} Viral replication in the hemopoietic cells in these cultures was documented, but the extent to which the gene of interest had been successfully transferred and expressed by primitive blood cell progenitors was not studied. Recently more direct quantitative data have been published by Hock and Miller¹² demonstrating retroviral-mediated gene transfer to primary human marrow progeni-

tors with recombinant viruses carrying the *neo'* or the DHFR* gene.

The present study was undertaken to investigate and identify variables that may influence the frequency of successful gene transfer to human hemopoietic progenitors. A number of the available viral packaging lines and retroviral vectors have been evaluated using a spectrum of target cell types including an established cell line as well as fresh progenitors of both normal and leukemic origin.

MATERIALS AND METHODS

Cells and culture conditions. Cell lines were cultured in Dulbecco's modified Eagle medium with high glucose (4.5 g/L) and 10% heat-inactivated calf serum (for ψ AM or ψ 2 cells) or 10% fetal calf serum for all other cell types in 5% CO₂ atmosphere at 37 °C. The amphotropic retrovirus packaging lines, ψ AM, PA12 and PA317, and the ecotropic packaging line, ψ 2, have been previously described.¹³⁻¹⁶

Primary human cells were obtained either from consenting adults (CML peripheral blood or normal bone marrow) or from second trimester abortions (fetal liver cells) after approval from the Clinical Screening Committee for Research Involving Human Subjects of the University of British Columbia. Blood and bone marrow cells were passed over a percoll density gradient (density 1.063) and light

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density cells collected and used in subsequent experiments. Fetal liver was minced with scissors and incubated for three hours at 37 °C in α medium with 20% fetal calf serum and collagenase 1 mg/mL (Sigma, St. Louis). The cells were then passed through a succession of needles of decreasing gauge, washed, and used for the experiments described.

Primary human cells were grown in α medium supplemented with 20% fetal calf serum and 10% agar-stimulated leukocyte-conditioned medium while in suspension culture and in methylcellulose assays as previously described¹⁷ for assessment for hemopoietic progenitors.

Virus production and assay. The general strategy for generating high-titer retroviral-producer cell lines was as outlined by Miller et al.¹⁸ The retrovirus packaging line, ψ 2, was transfected with viral plasmid DNA (pZipNeo or pN2).^{6,19} These plasmids are both MoMuLV-based vectors that contain the gene for neomycin phosphotransferase (neo^r). After 48 hours the medium from the transfected ψ 2 cells that contained neo^r virus (v-neo^r) was removed, and aliquots were used to infect the amphotropic packaging lines, ψ AM, PA12, or PA317. After 48 hours amphotropic v-neo^r-producing clones were selected in medium containing the neomycin analogue G418 at 1 mg/mL (Gibco Laboratories, Chagrin Falls, OH). G418 was dissolved in distilled water and added to growth medium to achieve the desired final concentration in total mg/mL (the effective drug concentration was approximately 50% of that value for the two lots of G418 used). Colonies were isolated by cloning rings, expanded, and examined for v-neo^r titer on 3T3 cells and for amphotropic helper virus using the S + L⁻ assay.¹⁵

Viral infection. The K562 human leukemic cell line or primary cells were infected with v-neo^r by either co-cultivation with amphotropic viral producer cells that had received 1,500 R irradiation or incubation in medium previously exposed to virus-producing cells for various periods of time. After the infection period they were maintained in suspension culture for 24 to 48 hours before plating in methylcellulose assay with or without G418. Control cultures that were not exposed to virus were grown in suspension culture and plated with or without G418 at the same time as the infected cells. All infections and control suspension cultures were done with polybrene, 8 μ g/mL. Colonies were scored after plating in methylcellulose on days 5 to 7 for K562 and days 10 to 14 for granulocyte macrophage colonies (from CFU-GM) and days 18 to 21 for large erythroid colonies (from BFU-E) for primary progenitor assays. Colonies were not scored unless they contained at least 30 cells and, in the case of BFU-E, had at least three clusters and were clearly hemoglobinized.

The infectious center assay was done by picking individual G418 resistant (G418^r) granulocyte-macrophage colonies from methylcellulose assay and placing the dispersed cells from one colony in a 2 cm² tissue culture well containing 10⁴ NIH-3T3 cells in medium with 8 μ g/mL polybrene. After overnight incubation the medium was replaced with fresh medium containing G418 1 mg/mL. Seven days later the assay was scored for the presence of G418^r 3T3 cells.

Cell and viral manipulations and cultures were performed under level C containment following Medical Research Council of Canada guidelines for handling retroviruses and human samples.

DNA and RNA studies. High molecular weight DNA was harvested from expanded clones of K562 cells and pooled hemopoietic colonies by proteinase K digestion followed by phenol and chloroform extractions. Southern blotting of K562 and primary hemopoietic colony DNA to nitrocellulose filters was performed by standard methods.²⁰

Total cellular RNA was harvested by the method of Meinkoth and Wahl²¹ from K562 or from primary hemopoietic colonies that had been individually plucked and pooled from methylcellulose

assays. The RNA was diluted to various concentrations and applied to nitrocellulose filters using a spot blot manifold according to established methods (Schleicher and Schuell, Keene, NH).

To detect neo^r sequences, a 2.3 kb neo^r-specific Bam HI, Hind III fragment isolated from RSV-neo plasmid DNA was ³²P-labeled by nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD) for use as a probe. Filters were prehybridized, hybridized, washed, and autoradiographed using standard techniques.²⁰

RESULTS

Viral producer cells. Many attempts were made to obtain the highest possible v-neo^r titer from various combinations of packaging lines and either the ZipNeo or the N2 vector. For the combination of ψ AM cells and pZipNeo, the highest titer of helper-free virus obtained after screening 61 clones was 2×10^4 CFU/mL. With PA12 and pZipNeo, several of the 21 clones studied produced approximately 10⁵ CFU/mL, but these were found to produce helper virus as well. With the combination of PA12 and the N2 vector, higher titers of v-neo^r (4×10^6 CFU/mL) were obtained, but these also contained helper virus. The highest titers of helper-free v-neo^r (5×10^5 CFU/mL) were obtained from the PA317 packaging cell line containing the N2 proviral DNA.

K562 experiments. To optimize conditions for high-frequency retroviral infection of human hemopoietic progenitors, we used K562 cells as a convenient model for experiments in which a number of variables were explored. Initially the best ratio of target K562 cells to irradiated viral producer cells was studied over a range of 10⁴:1 to 1:10. The transformation of K562 to G418^r increased in frequency as the proportion of v-neo^r producer cells increased up to a cell ratio of 1:1 and then plateaued with higher numbers of producer cells. A total of 10⁵ K562 cells were then incubated for two hours with 3 mL of medium exposed to v-neo^r producing cells, either undiluted or diluted to concentrations as low as 1:10³. There was a direct correlation between the concentration of v-neo^r-containing medium and the frequency of G418^r K562 cells for medium from all the viral producer lines tested. For the highest titer virus (4×10^6 CFU/mL) the frequency plateaued above concentrations of 1:10.

Figure 1 shows the frequency of G418^r K562 cells obtained by infection with undiluted v-neo^r-containing medium or co-cultivation with confluent cells of various types. The cell lines selected were those producing the highest v-neo^r titer for the various packaging line/neo^r plasmid combinations. At viral titers $\leq 10^5$ CFU/mL the maximum frequency of G418^r K562 cells was approximately 2% after co-cultivation with producer cells. Frequencies obtained after infection with medium exposed to the same cell lines was at least fourfold lower than that obtained by co-cultivation. At the highest titer, 4×10^6 CFU/mL, the difference in efficiency between co-cultivation and cell-free medium disappeared. The highest frequencies of G418^r K562 colonies were obtained with the PA12/N2 producer cells, approximately 60% for both co-cultivation with producer cells and incubation with medium.

A number of other variables that were investigated did not

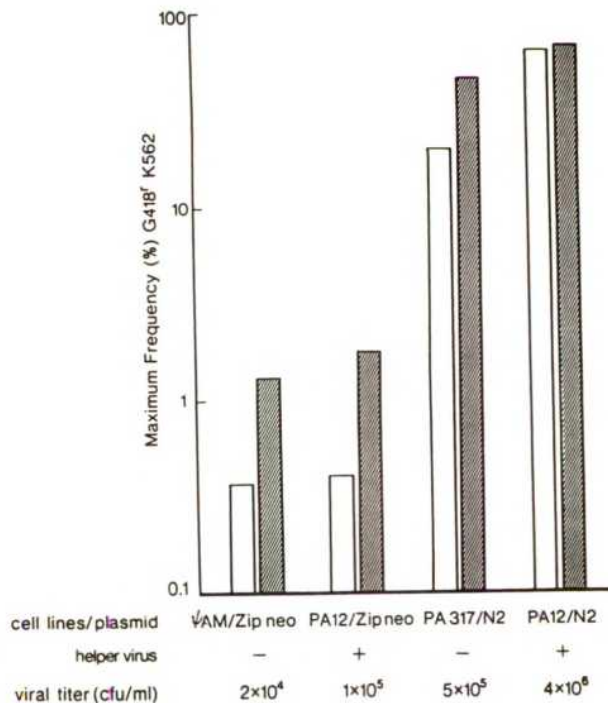


Fig 1. G418^r K562 colonies (% of colonies grown without G418) after infection of 10⁵ cells for two hours with v-neo^r from 3 mL undiluted medium or 24 hours exposure to a 60-mm dish of confluent, irradiated viral producer cells of various types. Each bar represents the results of a single experiment. Similar results were obtained in a second set of experiments. The G418 concentration of 1 mg/mL completely inhibited colony growth of uninfected cells. □, medium; ▨, co-cultivation.

appear to affect the frequency of K562 cell transformation to G418^r. These included extending the duration of co-cultivation from six to 48 hours or extending the duration of exposure to medium from producer cell lines from two to 48 hours.

The neo^r gene was demonstrated in G418^r K562 cell DNA by expanding individual colonies in liquid culture and extracting cellular DNA for Southern blots. Figure 2A demonstrates the 4kb neo^r-specific XbaI fragment found in four G418^r K562 colonies but not found in control K562 DNA. Figure 2B shows DNA from the same four G418^r K562 colonies and two uninfected control K562 colonies cut with enzymes that cut only once within the neo^r proviral DNA. The neo^r-specific fragments demonstrated in the G418^r colonies are single and unique to each colony, indicating one random site of integration for the provirus in the cellular DNA.

Infections of primary hemopoietic progenitors. A total of four normal marrow, six fetal liver, and six CML blood samples were infected with v-neo^r from a variety of packaging cell line/neo^r plasmid combinations. The infection procedure did not appear to be toxic to the target cells as shown by a lack of any effect on cell recovery or plating efficiency. (Values for infected cells were 80% to 100% of control values.) A G418 concentration of 2 mg/mL (effective drug concentration 1 mg/mL) completely inhibited all colony

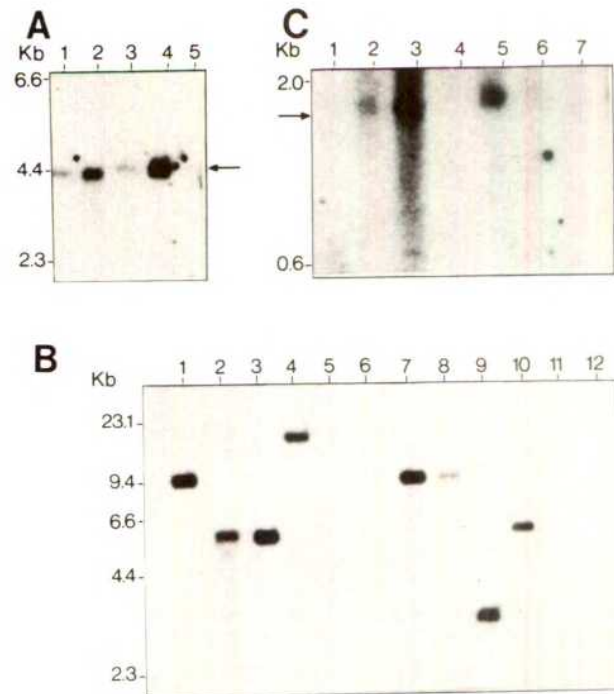


Fig 2. Southern blots of total cellular DNA hybridized with a ³²P-labeled neo^r-specific Bam H1-Hind III fragment from pRSV-neo. (A) K562 DNA from cloned G418^r cells infected with ψAM/ZipNeo viral stocks (lanes 1 to 4) or uninfected (lane 5) and digested with XbaI. → 4kb neo^r-specific fragment in infected cells. (B) DNA from the same G418^r K562 clones as in (A) (lanes 1 to 4 and 7 to 10) and uninfected clones (lanes 5, 6, 11, 12) digested with Bam HI (lanes 1 to 6), or EcoRI (lanes 7 to 12). These enzymes cut once within proviral DNA and once within cellular DNA to generate a single neo^r specific fragment of unique size in each of the G418^r clones. (C) DNA from pooled primary hemopoietic colonies either infected with PA12/N2 viral stocks (lanes 2, 3, 5) or uninfected (lanes 1, 6, 7) and digested with EcoRI. The expected 1.5 kb neo^r specific fragment is seen in lane 2 (cells infected with viral supernatant but not selected in G418), lane 3 (G418^r cells after infection with supernatant), and lane 5 (cells infected by co-cultivation with producer cells but not selected in G418). Lane 4 shows no neo^r specific hybridization from cells exposed to supernatant from PA317/N2 producer cells but not selected in G418.

growth in assays of uninfected primary progenitors. No difference in G418 sensitivity between the various target cell sources or between progenitors of different lineages (ie, BFU-E and CFU-GM) could be demonstrated.

Figure 3A shows the frequency of G418^r CFU-GM seen after infection by v-neo^r producer cells at various viral titers. Each point represents a different experiment combining target cells of a certain type with a specific viral producer cell line. Although there was a correlation between viral titer and frequency of G418^r, considerable variation in transformation efficiency at any given titer was also found; for example, from 2.4% to 15.7% G418^r CFU-GM at a titer of 4 × 10⁶ CFU/mL. There was also a low (0.045% to 0.4%) but reproducible incidence of G418^r colonies after infection with cells producing viral titers as low as 10³ CFU/mL.

Figure 3B shows similar data for BFU-E. Infections with cells producing a v-neo^r titer of 1 × 10³ CFU/mL yielded a

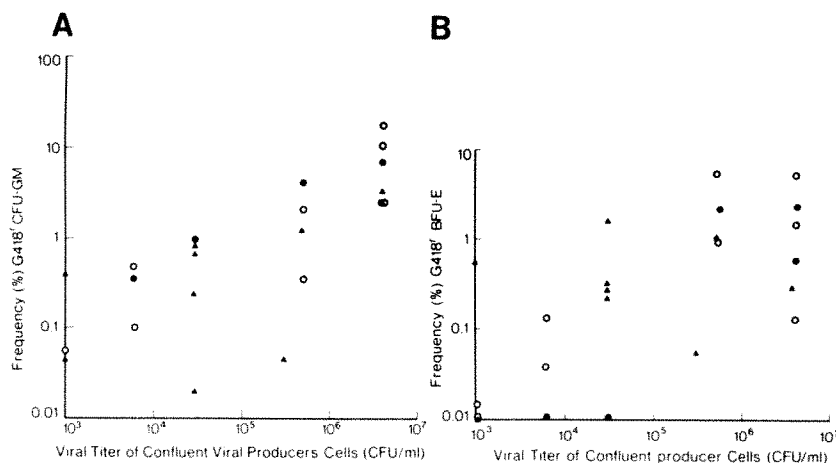


Fig 3. Frequency (%) of G418^r primary hemopoietic colonies (G418^r colonies/total colonies without G418 × 100) after 24-hour exposure of 5×10^6 cells to a 60-mm dish of irradiated, confluent v-neo⁺ producer cell lines at various viral titers. ●, normal bone marrow; ○, CML blood; ▲, fetal liver. (A) Granulocyte, macrophage colonies; CFU-GM. (B) Large erythroid colonies; BFU-E.

frequency of large G418^r erythroid colonies of less than 1%, while frequencies up to 5.6% were seen after infections done with cell lines producing higher titers. However, the correlation of G418^r frequency with titer was less clear, and the variation in G418^r frequency at any given titer was similar to that seen for CFU-GM. From these data (Figs 3A and B) it appears that BFU-E may be less readily transformed by v-neo⁺ than CFU-GM. Although this may be true, it is possible that the apparent difference is the result of experimental variables that particularly affect erythroid colony growth. For example, it was found that in general G418^r erythroid colonies were not as large or as red as those obtained from infected BFU-E plated in the absence of G418. It is possible that impurities in the G418 affect the growth of erythroid colonies containing the neo^r gene. Alternatively, the neo^r gene may be expressed at a lower level in erythroid cells as they differentiate. Therefore it is likely that the frequencies recorded here for G418^r BFU-E represent minimum estimates of successful gene transfer.

The maximum frequencies of G418^r progenitors from these experiments are summarized in Table 1. The highest frequency was usually obtained after infection with the cell line producing the highest viral titer, PA12/N2 cells producing v-neo⁺ at 4×10^6 CFU/mL, in addition to helper virus.

The ability of medium exposed to v-neo⁺-producing cells,

as compared to co-cultivation with producer cells of various types, to transform primary progenitors to G418^r is compared in Fig 4. At a titer of 6×10^3 CFU/mL produced by ψ AM/ZipNeo cells, v-neo⁺-containing medium was unable to produce a significant incidence of G418^r colonies while co-cultivation yielded a frequency of approximately 0.1%. At titers of 5×10^5 or 4×10^6 CFU/mL, medium with virus was effective at transforming both BFU-E and CFU-GM. However, the frequency was still two- to 50-fold higher using co-cultivation. Nevertheless, the frequency of G418^r colonies was higher after infection with medium at high titers (10.6% for CFU-GM at 4×10^6 CFU/mL) than with co-cultivation at low titers (0.13% CFU-GM at 6×10^3 CFU/mL).

The frequencies of transformation for primary progenitors were not as high as those obtained for K562 cells. To try to increase the efficiency, target cells were allowed to remain on viral producer cells for as long as seven days without any increase in the efficiency of transformation.

The ability of G418^r CFU-GM that had been infected with helper-containing v-neo⁺ to infect 3T3 cells was tested by infectious center assay. In two experiments, five of 22 and 24 of 24 individual CFU-GM tested transformed 3T3 cells to G418 resistance. The production of G418^r 3T3 cells in this assay requires that the infecting cells be producing v-neo⁺. To do so they themselves must have been infected by both v-neo⁺

Table 1. Maximum Frequency of G418^r 1⁰ Hemopoietic Colonies After Co-cultivation With Various v-neo⁺ Producer Cells

Target Cells	Viral Producer Cells	v-neo ⁺ Titer (CFU/mL)	G418 ^r Colonies/Colonies Without G418 (%)					
			CFU-GM		BFU-E			
			+ Virus	Control†	+ Virus	Control†	+ Virus	Control†
Normal Marrow	ψ AM/ZipNeo	3×10^4	26/2775	(0.94)	0/2970	—	—	—
	PA317/N2	5×10^5	78/1860	(4.2)	0/630	85/3930	(2.2)	0/4050
	PA12/N2*	4×10^6	127/1800	(7.1)	0/630	101/4260	(2.4)	0/4050
Fetal Liver	ψ AM/ZipNeo	3×10^4	24/2960	(0.81)	0/3996	54/3333	(1.6)	0/18468
	PA317/N2	5×10^5	2/158	(1.3)	0/150	1/105	(1.0)	0/120
	PA12/N2*	4×10^6	13/414	(3.1)	0/150	2/690	(0.31)	0/120
CML Blood	ψ AM/ZipNeo	6×10^3	48/9900	(0.48)	0/6600	18/14320	(0.13)	0/9523
	PA317/N2	5×10^5	66/3120	(2.1)	0/3888	194/3480	(5.6)	0/6480
	PA12/N2*	4×10^6	656/4170	(15.7)	0/16920	1672/31650	(5.3)	0/81180

* + Helper virus.

†Control = Cells from the same sample plated in methylcellulose $\pm$ G418 without exposure to v-neo⁺ producer cells.

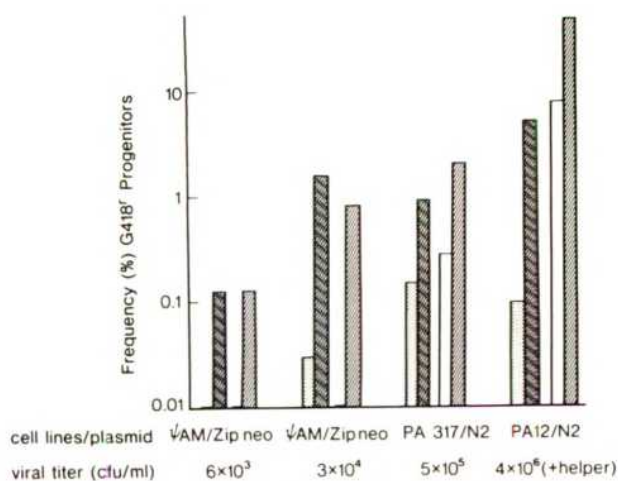


Fig 4. Frequency (%) of G418^r primary hemopoietic progenitors (G418^r colonies/total colonies without G418 × 100) after infection of 5×10^6 cells by v-neo^r from a 60-mm dish of irradiated confluent producer cells for 24 hours or 5 mL undiluted medium of various types for two hours. Each bar represents the results of one experiment. For each viral titer the target cells were from the same individual for all conditions studied. At titers of 5×10^5 and 4×10^6 CFU/mL, the same CML blood cells were used. CML cells were also used at 6×10^3 CFU/mL and fetal liver cells at 3×10^4 CFU/mL. Similar results were obtained in a second set of experiments. BFU-E exposed to v-neo^r medium, □; BFU-E exposed to confluent producer cells, ▤; CFU-GM exposed to v-neo^r medium, □; CFU-GM exposed to confluent producer cells, ▤.

and helper virus, which may occur relatively infrequently.¹² This would account for less than 100% of G418^r CFU-GM being positive in the infectious center assay.

Figure 2C is a Southern blot of DNA harvested from pooled hemopoietic colonies grown from CML blood cells that were either uninfected or exposed to viral stocks containing v-neo^r. After digestion with EcoRI, the neo^r-specific probe identified the expected 1.5 kb neo^r-specific fragment in G418^r cells (lane 3). A less intense signal is seen in lane 2 where the same cells were exposed to medium containing v-neo^r but not selected in G418, indicating that some of the cells did not contain the neo^r gene (8% of the CFU-GM in this sample were G418^r). In lane 5 the DNA from cells exposed to viral producer cells but not selected in G418 shows a strong signal. In colony assays 15.7% of the CFU-GM in this sample were G418^r. The strength of the hybridization signal in lane 5 as compared to lane 3, where an equivalent amount of DNA was hybridized, indicates that at least 15.7% of the cells in this sample contained the neo^r gene. DNA from cells from the same sample that were not exposed to virus in lanes 1, 6, and 7 show no signal.

Expression of the neo^r gene by v-neo^r infected, pooled primary progenitors or K562 cells was shown by RNA spot blot (Fig 5). Total cellular RNA was hybridized with a neo^r-specific probe. Uninfected, control cells show no evidence of neo^r hybridization, while the same number of infected cells show a strong signal. The signal is strongest for cells that were selected in G418 (lanes b, CFU-GM and all infected K562 cells) but is also seen in cells that were infected but not selected (lanes a, CFU-GM).

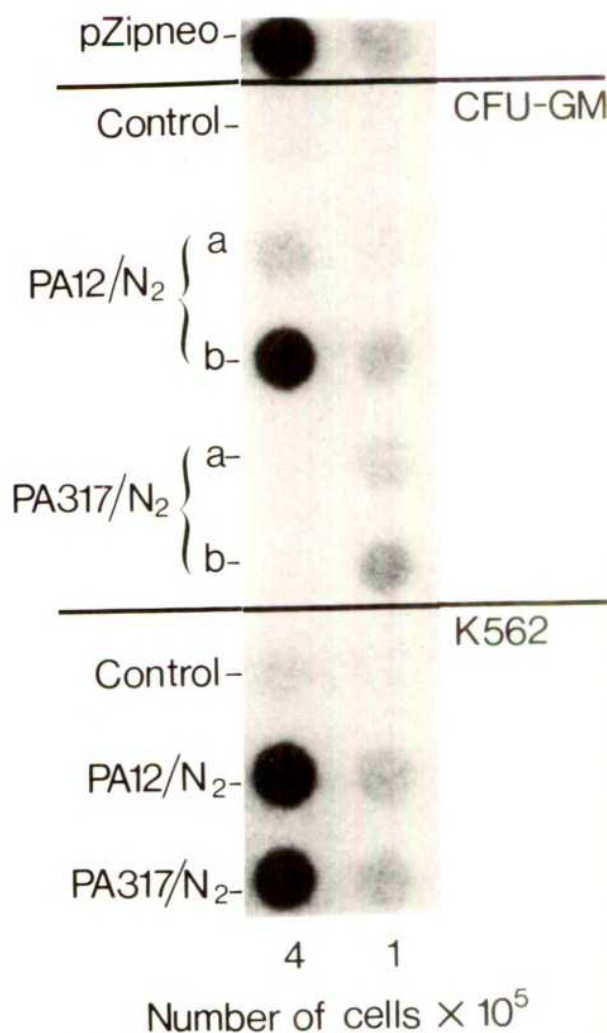


Fig 5. RNA spot blot of total cellular RNA from pooled CML granulocyte-macrophage colonies or K562 cells hybridized with a ³²P-labeled neo^r specific Bam HI-Hind III fragment from pRSV. Control cells were not exposed to v-neo^r or G418. Cells exposed to either PA12/N2 or PA317/N2 v-neo^r producer cells were either selected in G418 (b) or unselected (a) in the case of CFU-GM. Insufficient cells were available to allow samples for the PA317/N2, 4×10^5 cells column. Therefore, these two spots are blank. All infected K562 cells were selected in G418. pZipNeo DNA serves as a positive control in the top lane (2.0 and 0.5 pg DNA from left to right).

DISCUSSION

These data demonstrate efficient transfer and expression of the neo^r gene to both the K562 human leukemic cell line and a variety of normal and malignant primary human hemopoietic progenitors using recombinant retroviruses.

A number of variables were investigated to try to achieve the highest possible efficiencies of gene transfer. Co-cultivation with viral producer cells rather than infection with medium exposed to viral producer cells and use of high titer virus were both identified as important parameters, but viral titer clearly had the greatest influence on the frequency of transformation to G418 resistance. Considerable effort was

devoted to studying viral producer cells generated by various packaging line/*neo'*-containing plasmid combinations. The choice of these variables had a major impact on the titer of both v-*neo'* and helper virus. We were unable to generate high titer v-*neo'* from the PA12 packaging line that was not associated with helper virus. The use of the N2 vector rather than ZipNeo raised the v-*neo'* titer but did not eliminate the problem of helper virus. Although the PA12 line has been constructed to generate helper-free recombinant virus, it appears that with the vectors used in this study sufficient genetic recombination occurs between the defective packaging and recombinant *neo'* viral sequences to frequently generate significant helper virus titers. Helper-free v-*neo'* generated by the ψ AM packaging line containing pZipNeo DNA was always of relatively low titer ($<10^5$ CFU/mL). The newer generation of retroviral packaging lines, such as the PA317 developed by Miller and Buttimore,¹⁶ may solve the problem of obtaining high-titer recombinant virus without associated helper.

Although both ZipNeo and N2 contain the same *neo'* gene under the control of the promoter in a Moloney leukemia virus LTR, the two vectors are not identical in structure.^{6,19} Although the differences appear subtle, they seem to affect the viral titers obtained from producer cells and might also cause different levels of expression of the transferred foreign gene in infected hemopoietic cells. For this reason we elected to test virus derived from both the N2 and ZipNeo vectors on our target cells. Although our data are not definitive, some of our results (eg. Fig 1 where K562 were the target cells and a fivefold increase in viral titer led to a 20-fold increase in frequency of G418^r colonies) may be explained if *neo'* was expressed more efficiently in cells infected with virus derived from N2 rather than ZipNeo.

In experiments done with helper-free virus from a producer line derived from PA317 cells and the N2 plasmid, we were successful in demonstrating gene transfer at levels only slightly lower than those achieved with helper containing virus of significantly higher v-*neo'* titer. No obvious effect of helper virus on the frequency of gene transfer was detected.

In addition to normal bone marrow we evaluated two alternative sources of primary human progenitors for retroviral infections. These were fetal liver and CML blood. In both cases a high proportion of the primitive progenitors in these tissues are in the active part of the cell cycle as compared to their counterparts in normal marrow, which are largely quiescent.²² Cell cycling status has been suggested as an important variable affecting the success of retroviral infection and integration into host DNA.²³ Although some of our highest frequencies of G418^r colonies were seen in the CML blood cultures, there was sufficient variability from experiment to experiment that no overall difference was evident. However, there was clearly no dramatic improvement in the frequency of G418^r progenitors in experiments using either of the primary human targets that were presumably cycling optimally.

There are many possible reasons for the variability in the frequency of successful gene transfer and expression in primary progenitors, as illustrated in Fig 3. Although the viral titer from our v-*neo'* producer cell lines was quite stable

over long periods of time, some differences did occur. For example, after the producer cells reach confluence the viral titer begins to drop significantly. Similarly, the ability of target cells not only to become infected but to integrate and express the viral genome is probably affected by a number of factors, including their level of differentiation, cell cycle status, and perhaps subtle metabolic factors that may be altered by the unavoidable day-to-day changes in complex culture conditions. Although care was taken to ensure that both viral producer and target cells were in optimal condition at the time of infection, some variability undoubtedly occurred and may account for changes in frequency of G418^r colonies. For example, the fetal liver samples underwent considerable manipulation, and the plating efficiency of uninfected cells in progenitor assays was highly variable. Changes affecting the ability of hemopoietic progenitors to form colonies in culture might even more profoundly reduce the frequency of successful gene transfer. If so, this would account for data such as the low numbers of G418^r BFU-E after infection of fetal liver cells with virus from PA12/N2 producer cells (Table 1).

Although encouraging, the frequency of gene transfer demonstrated to primary progenitors by these data is still much lower than the theoretical 100% that should be obtainable with retroviruses.¹ Our frequencies are reasonably close to those reported by Hock and Miller for gene transfer to primary normal human hemopoietic progenitors from bone marrow¹² but are much lower than the 75% to 100% frequency reported by Eglitis et al for murine CFU-S.⁶ However, it is notable that the latter authors and others have found the level of expression of G418 resistance or *neo'* gene product to be highly variable among different CFU-S containing the *neo'* gene.⁴⁻⁶ The frequencies of G418 resistance in murine CFU-GM infected and selected *in vitro* have been approximately 10% to 30%,^{4,5} which is also lower than one might have predicted and consistent with our results on human progenitors.

A large number of techniques exist for transporting genetic material into cells. A number of these have been applied to primary hemopoietic progenitors, including calcium phosphate co-precipitation and electroporation.²⁴⁻²⁶ Although initial results using the former technique to confer methotrexate resistance on murine bone marrow cells appeared promising, similar results have not been forthcoming for other genes or for human cells. Electroporation is a relatively new technique that has not been widely applied to gene transfer into primary cells. Initial results with human bone marrow progenitors demonstrate low levels of transfer and expression of the xanthine-guanine phosphoribosyl transferase gene in CFU-GM.²⁶ The full potential of this technique remains to be explored. At the present time the bulk of evidence would indicate that the highest efficiencies of gene transfer are those mediated by retroviruses. Where high frequencies of transformation are necessary and appropriate vectors are available, these viruses appear to be the current method of choice for transporting genes into cells.

The knowledge gained from the present studies should facilitate the successful use of the technique of retroviral-mediated gene transfer to study genetic factors important in

normal and malignant hemopoiesis. In addition, there are a number of inherited human hematologic disorders that are known to have a molecular genetic etiology. Some of these may ultimately be curable by gene replacement therapy, possibly with retroviruses providing the means of transporting the normal gene into primitive hemopoietic progenitors.²⁷

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Structural Polymorphism of Glycophorins Demonstrated by Immunoblotting Techniques

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We have explored the polymorphism of the glycophorin system in the human erythrocyte membrane using the immunoblotting techniques and examining 52 individuals selected without prior bias as to their serologic state and ten documented serologic variants of M, N, S, s blood group system. Polyclonal antisera to α glycophorin and to α glycophorin CNBr carboxyl terminal fragment C (residues 82-131) and M and N specific monoclonal antibodies (MoAbs) were used. The first two reagents detect specific regions of the α glycophorin molecule and all electrophoretically resolved species of glycophorins immunologically related to α and δ glycophorins (δ glycophorin, [α - δ] hybrids and other glycophorins with an alteration in the carboxyl terminal segment); the M and N MoAbs identified the glycophorin species containing or lacking the M or N

determinant in the amino terminal octapeptide structures. We find that (1) immunoblotting confirmed in all cases the serologically determined phenotype; we also find that polymorphic forms of the glycophorin system are relatively infrequent; (2) immunoblotting, independent from serologic testing, was capable of detecting five mutants, two most like γ S-s-U-phenotypes; (3) a new glycophorin species was detected in normal red cells with both antiglycophorin and antipeptide C sera, which is not evident with MoAbs; (4) immunoblots of known glycophorin variants (En(a-), U-, M^a, Mi I, II, III, V, and St^a) confirmed but also extended our knowledge of the abnormal glycophorins involved; and (5) the He⁺ and Wr^b(-) cells showed normal patterns.

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GLYCOPHORINS are a family of erythrocyte membrane glycoproteins, and in each individual several related forms are present.^{1,2} In the major M and N α glycophorins,^{*1} the antigens that determine the M, N blood groups reside; these glycoproteins differ in two amino acids at residues 1 and 5 of the polypeptide chain and are products of two codominantly expressed allelic genes.¹⁻⁶ Moreover, δ glycophorins, in which the S and s blood group antigens reside, are specified by other allelic genes⁶⁻⁸; these relatively less abundant glycophorins are related to the N form of α glycophorin but show amino acid sequence differences and lack the asparagine-linked oligosaccharide and the carboxy terminal segment of α glycophorin.^{1,7-9} The β and τ glycophorins are minor components whose serologic and structural characteristics are less clear.^{1,2}

As shown by recent gel electrophoretic and structural

studies, erythrocytes of serologic variants of the MNSs locus may exhibit additional variant forms of glycophorins. Blood group specific M^a, M^c, Mi I, and II erythrocytes contain products of variant alleles of the M or N genes,¹⁰⁻¹³ and those of St^a, Miltenberger V, or Ph individuals contain fusion glycophorins originating from recombination events between α and δ glycophorin genes.¹⁴⁻¹⁸ Some individuals and their families are known whose erythrocytes do not bear either α (En(a-)) or δ glycophorins (S-s-U-)^{1,2}; and others have cells that contain α glycophorin glycosylation variants, for example the Cad phenotype.^{19,20}

In this report we examined two aspects of glycophorin polymorphism using immunoblotting approaches. First, the extent of occurrence of glycophorin variants in a population of 52 individuals, selected without prior bias as to their serologic state; and second, the ability to be detected and the nature of profile differences in erythrocytes of individuals with serologically defined variant M, N, S, and s phenotypes.

MATERIALS AND METHODS

Preparation of erythrocytes. Random control erythrocytes were obtained from laboratory personnel and from 41 medical students from Central Africa (Cameroon, Zaïre, and Central African Republic).^{†2} Venous blood (collected with heparin or citrate-phosphate-dextrose) was centrifuged at 3,000 rpm for 30 minutes and plasma and buffy coat removed by aspiration. The erythrocytes were washed three times with isotonic phosphate buffer pH 7.6, followed by two washes with the same buffer containing inhibitors of proteolysis: 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 2 mmol/L N-ethyl maleimide, and 0.02% sodium azide. Washed cells were centrifuged at 2,000 rpm for 15 minutes and used for blood typing and gel electrophoretic studies.

Serologically variant erythrocytes were obtained from individuals referenced in the rare donor file at New York Blood Center; their phenotypes are noted in Table 1. These cells had been frozen in

*Glycophorins will be designated essentially as proposed by Anstee²; α glycophorin is equivalent to glycophorin A or PAS-1 and PAS-2 glycoproteins, δ glycophorin is equivalent to glycophorin B or PAS-3; γ glycophorin is equivalent to glycophorin C and glycoprotein E (See Fig 1).² Fusion hybrids are glycophorins in which the amino terminal portion of the molecule has a sequence identical to one type of glycophorin, whereas the carboxyl terminal portion arises from another type of glycophorin. Unequal crossing over may be a genetic mechanism involved in the generation of these fusion hybrids.

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[†]The original intention for the collection of bloods from these subjects was that they serve as controls in an unrelated sickle cell hemoglobin study; their MNSs serologic state was unknown to us at the time of collection.

Table 1. Reaction of Variant Glycophorins in Erythrocytes with Rare Serologic Variants of M, N, S, s With Specific Polyclonal Antisera and MoAbs

Serologic Phenotype	Variant Bands*	Reaction With				Possible Structural Characteristics†
		Antisera to		MoAbs		
		MN Glycophorin	Peptide C	M	N	
MiI, M, N, S, $\bar{s}$	4'	+	+	—	—	Glycophorin variant altered in the amino terminal octapeptide
Mill, M, S, $\bar{s}$	4'	+	+	+	—	Glycophorin variant (M-like)
Mill, N, S	2'	+	+	—	+	δ Glycophorin variant
	2''	+	—	—	+	
	4'	+	—	—	+	
MiV, M, N, $\bar{s}$	2'	+	—	—	+	δ Glycophorin variant
	2''	+	+	—	+	
	2'''	+	—	—	+	
	4'	+	—	—	+	
St ^a , M, N, $\bar{s}$	2'	+	+	—	+	α Glycophorin variant
	2''	+	+	—	+	
	2'''	+	+	—	+	
En(a—)(Fin), S, $\bar{s}$	Bands 1 and 4	—	—	—	—	Lack α glycophorin; "normal" δ glycophorin
M ^a M ^b , $\bar{s}$	Bands 1 and 4	+	+	—	—	α glycophorin altered within the amino terminal octapeptide; "normal" δ glycophorin
U [—] , N	Band 6	—	—	—	trace	lack δ glycophorin; "normal" α gly- cophorin
He ⁺ , M, S, $\bar{s}$	No obvious differences from normal.					
W ^b , M, N, $\bar{s}$						

*See Figs 1 to 3;

†Structural characteristics apparent from reaction with the antisera and the antibodies.

liquid nitrogen and were reconstituted by adding a few frozen droplets at a time to 5 mL of saline solution at 45 °C.^{21,22} The cells were washed three times in saline and processed as described above for the freshly collected cells.

Blood group typing. Hemagglutination was performed according to standard procedures using commercial rabbit anti-M and anti-N sera (Ortho Diagnostic Systems, Raritan, NJ).

Polyacrylamide gel electrophoresis. Gel electrophoresis was carried out on 10% to 15% gradient gels in 0.1% sodium dodecyl sulfate (SDS) with a 3% acrylamide stacking gel as described by Laemmli.²³ Erythrocytes were prepared for electrophoresis as follows: to 0.025 mL of packed cells was added 0.5 mL of distilled water, followed by 0.25 mL of 3x sample buffer.²³ The solution was immersed for one minute in boiling bath and 10 μ L loaded into each of 20 slots of the slab gel. The remaining solution, in 0.1-mL aliquots, was kept at –20 °C. No change in electrophoretic patterns was observed on replicate analyses of the frozen samples.

Western immunoblotting. Immunoblotting was performed as described by Towbin et al.²⁴ Following electrophoresis the proteins in the unstained gels were electrophoretically transferred to nitrocellulose sheets in a Trans-Blot Cell (Bio-Rad) at 100 mA for 16 to 18 hours, using a transfer buffer consisting of 50 mmol/L Tris, 192 mmol/L glycine, and 20% (wt/vol) methanol. The blots were blocked with 7% bovine serum albumin, rinsed with 0.05 mol/L Tris-HCl-0.15 mol/L NaCl (pH 7.6), and incubated for one hour at room temperature with the appropriate antibodies, either antiglycophorin serum (1:200 dilution), antiserum to CNBr carboxyl-terminal fragment C (1:200 dilution), M-specific monoclonal antibody (MoAb) (1:5 dilution of culture supernatant fluid), or N-specific MoAb (1:100 dilution of purified immunoglobulin from ascites fluid, 1 mg/mL). In the case of the MoAbs the nitrocellulose

sheets were first exposed to rabbit antimouse IgG (1:750 dilution). One per cent bovine serum albumin was used for all dilutions. The nitrocellulose sheets were washed with 1% Tween in the above buffer and incubated with ¹²⁵I-Protein A prepared as described.²⁵ Following extensive washing with the Tween-buffer solution, the nitrocellulose blots were exposed to Kodak XAR-5 film for 1.5 to 3.0 days.

Preparation and properties of antisera and antibodies. Antisera were prepared in rabbits against α glycophorin prepared from erythrocyte membranes of an MN individual and against carbohydrate-free carboxyl terminal peptide C (residue 82–131) obtained by CNBr cleavage of α glycophorin.^{18,26} Both antisera react with M, N α glycophorin and its carboxyl terminal peptide C but do not react with the internal carbohydrate-rich portion of α glycophorin (CNBr peptide B, residues 9–81).¹⁸ Preimmune rabbit sera showed no bands when tested with purified glycophorins; with preparations of intact M, N, or MN erythrocytes a very faint band was seen at the position of band 5 similar in intensity to that seen in En(a—) cells (Fig 1; Ena(—) lanes A and B). More generally the specificity of the antisera is demonstrated by the pattern of reaction with the Ena(—) cells (lane B).

An M-specific MoAb (1–2–D9) was produced as previously described for MoAbs G8 and E₃.²⁷ The N-specific MoAb (NN 5) has been previously described.²⁸ The M MoAb reacted exclusively with the M α glycophorin and the N MoAb reacted with N α and δ glycophorins when tested on Western immunoblots with M and N α glycophorins isolated from MM or NN erythrocytes.¹⁸ The specificities of antisera and antibodies are summarized in Table 2. Preimmune mouse serum showed a faint band slightly below the position of band 2 and a much stronger band at the position of band 5 when tested with MN or NN erythrocytes (see Fig 1 for designation of glycophorin bands).

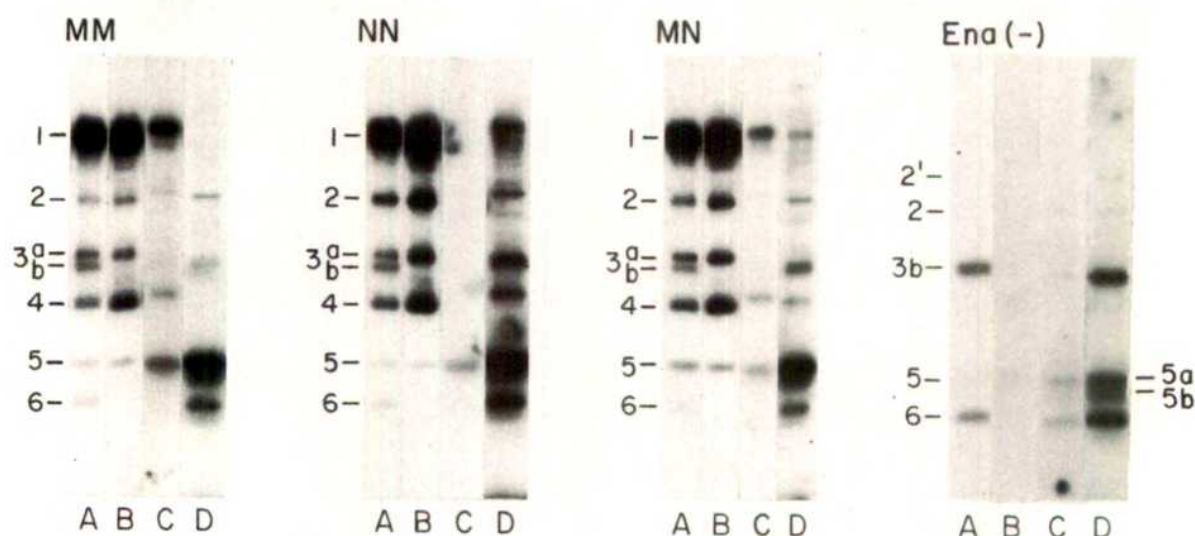


Fig 1. Fluorographs of immunoblots of erythrocytes of serologically "normal" MM, NN, MN representative individuals and an individual of the uncommon En(a-) phenotype. Lanes A, treated with antiglycophorin serum; Lanes B, with anti-peptide C serum; Lanes C, with M MoAb; Lanes D, with N MoAb. Identical aliquots of solubilized erythrocytes from each individual were subjected to electrophoresis on a single or different slab gel that were then processed as described. The fluorograms were photographed and lanes obtained with the respective antisera or antibodies were cut out and collated to display for each individual the reaction under each of the four conditions (lanes A to D). When electrophoresis was carried out on different slab gels, the mobility of bands was close but not always identical; for example, in En(a-) cells, lanes A and D are not exactly coincident. The numbers on the left identify bands that react with antiglycophorin serum. Bands denoted by the prime symbol ' are those that were not seen in serologically "normal" individuals. Replicate analyses of erythrocytes of each individual were carried out with all four reagents. In all cases identical patterns were obtained when conditions described in the text were used.

RESULTS

Glycophorins in erythrocytes of MN individuals. Erythrocytes of random individuals displayed seven bands on SDS-polyacrylamide gels when probed with the polyclonal antiserum to α glycophorin. This is shown in Fig 1 (lanes A), for erythrocytes of three representative MM, NN, and MN

individuals of 52 individuals examined. Bands 1 and 4 correspond to the α glycophorin dimer and monomer, respectively; bands 3b and 6 migrate in the area of δ glycophorin dimer and monomer, and band 5 corresponds to γ glycophorin (Table 3). In addition to these well-established bands,² an

Table 2. Specificity of Polyclonal Antisera and Monoclonal Antibodies

	Reaction With	No Reaction With
Antiserum to MN α glycophorin	α , δ glycophorins and other minor glycophorins	CNBr peptide B*
Antiserum to carboxyl terminal peptide C of α glycophorin (residue 82-131)	α glycophorins and all other glycophorins related to α glycophorins in the carboxyl terminal	δ glycophorins and all glycophorins altered or lacking the carboxyl terminal region of α glycophorins CNBr peptide B*
M-specific MoAb	M α glycophorin† (ser ser thr thr gly val ala met)	N α glycophorin δ glycophorin
N-specific MoAb	N α glycophorin† δ glycophorin (leu ser thr thr glu val ala met)	M α glycophorin

*Blumenfeld et al¹⁸

†Epitope is located within the amino terminal octapeptide as shown.

‡ = O-linked saccharides; Blumenfeld and Adamany³

Table 3. Reaction of Glycophorins in Erythrocytes of Randomly Chosen Individuals with Specific Polyclonal Antisera and MoAbs

	Reaction with			
	Antisera to		MoAbs	
	MN α glycophorin	peptide C	M	N
Bands 1 and 4*				
α glycophorin dimer and monomer	+	+	According to serologic phenotype	
Bands 3b and 6				
δ glycophorin dimer and monomer	+	-	-	+
Band 2				
α - δ dimer?	+	+	+	+
Band 5				
γ glycophorin and possibly other glycophorins	+	+	+	+
Band 3a				
glycophorin X	+	+	-	-

*See Fig 1.

additional band designated 3a is seen. β -Glycophorin bands, noted by others,² were not visualized and probably co-migrate with the monomer of α glycophorin (band 4).

As shown in Fig 1 (lane B) and Table 3, the antiserum to the carboxyl-terminal peptide C does not react with δ glycophorin in bands 3b and 6 but reacts with the other glycophorins. This confirms that δ glycophorin differs from α glycophorin either by an alteration in the carboxyl-terminal region or by the absence of that region. This also suggests that the minor glycophorins in bands 2, 3a, and 5 are structurally related with respect to that polypeptide region to α rather than to δ glycophorin.

The monoclonal M- and N-specific antibodies react with α glycophorins according to the M or N serologic phenotype of the cells; in addition, the N-specific MoAb reacts with δ glycophorins, even in the M positive cells (Fig 1, lanes C and D, Table 3). It was observed that in all the erythrocyte samples tested, band 3a was not demonstrated by either MoAb whereas band 5 was evident with both. The reactivity of pre-immune mouse serum at band 5 but not at bands 1 and 4, for example, suggests a specific antigen/antibody reaction rather than a nonspecific binding effect. It would appear therefore that some of the glycophorins recognized by pre-immune mouse serum also carry M- and N-specific epitopes recognized by the monoclonal reagents.

The glycophorin band profiles observed were all essentially similar for the 52 randomly chosen individuals. The only deviations observed were the absence of δ glycophorin in two individuals and the absence of band 5 using anti-peptide C serum in three individuals. The latter observation suggests that a structural alteration exists in the carboxyl terminal region of these glycophorins. The erythrocytes of the two former individuals could possibly exhibit the S- s- U- blood group phenotype. Unfortunately, fresh red cells were not available for confirmatory blood typing tests to be performed.

Glycophorins in erythrocytes with variant serologic phenotypes. Erythrocytes from ten different individuals exhibiting variant or null glycophorin phenotypes were examined

(Table 1). The altered glycophorin profiles became apparent when the cells were probed with the polyclonal antibody to α glycophorin (Figs 1 to 3). Thus in En(a-) cells the usual α glycophorin bands were absent, and in U- cells the δ glycophorins were absent; and in Mi I, II, III, V, and St^a cells, variant glycophorins (denoted as prime, ') could readily be detected by their electrophoretic mobilities, which were distinct from the usual glycophorin bands. In the case of M^a homozygous erythrocytes, the variant nature of α glycophorins was apparent because they did not react with the M- or N-specific MoAbs. These observations, summarized in Table 1, are essentially in agreement with reported findings.^{1,2,10-18,29,30}

Additional aspects of the structure of the altered glycophorins were apparent from the known specificities of the antisera and antibodies used. For example, the variant glycophorin in band 4' in MiII erythrocytes did not react with either M or N MoAb; in contrast, this band was distinctly evident with MiII cells and the M MoAb (Fig 2). Clearly the amino terminal region of this glycophorin in Mi I and Mi II cells is different. Bands 3b and 6 in En(a-) erythrocytes appear to contain the dimer and monomer of δ glycophorin from their reaction with antiglycophorin serum, the N MoAb, and lack of reaction with anti-peptide C serum. In addition to having no α glycophorin, these cells also lack the glycophorins in bands 2 and 3a seen in "normal" cells. However, at the position of band 5 these erythrocytes contain a glycophorin that reacts with both M and N MoAbs (a double band is seen with the N MoAb; Fig 1). Glycophorins in bands 2'' and 4' in Mi III cells resemble δ glycophorin in that they fail to react with anti-peptide C serum. They are probably variant forms of δ glycophorins whose usual 3b and 6 bands are less evident and appear to react with anti-peptide C serum.

Glycophorin bands 2', 2'', and 4' in Mi V cells appear to be variant forms of δ glycophorin, as they do not react with anti-peptide C serum; indeed it has been proposed that they are (α - δ) glycophorin hybrids.¹⁶ In contrast, St^a glycophorin variant bands 2', 2'', and 2''' react with anti-peptide C serum

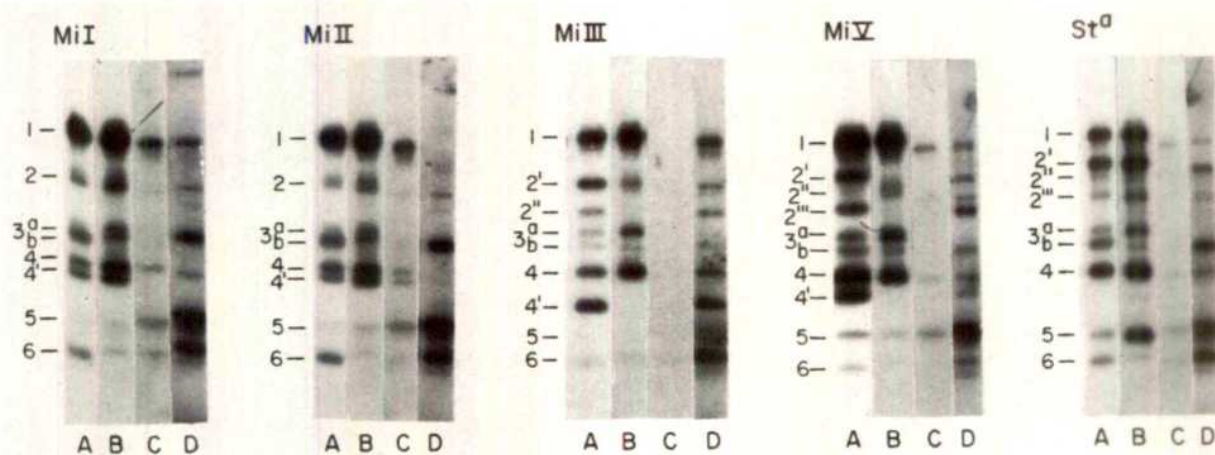


Fig 2. Fluorographs of immunoblots of variant MiI, MiII, MiIII, MiV, and St^a erythrocytes. Lanes A, treated with antiglycophorin serum; lanes B, with anti-peptide C serum; lanes C, with M MoAb; lanes D, with N MoAb.

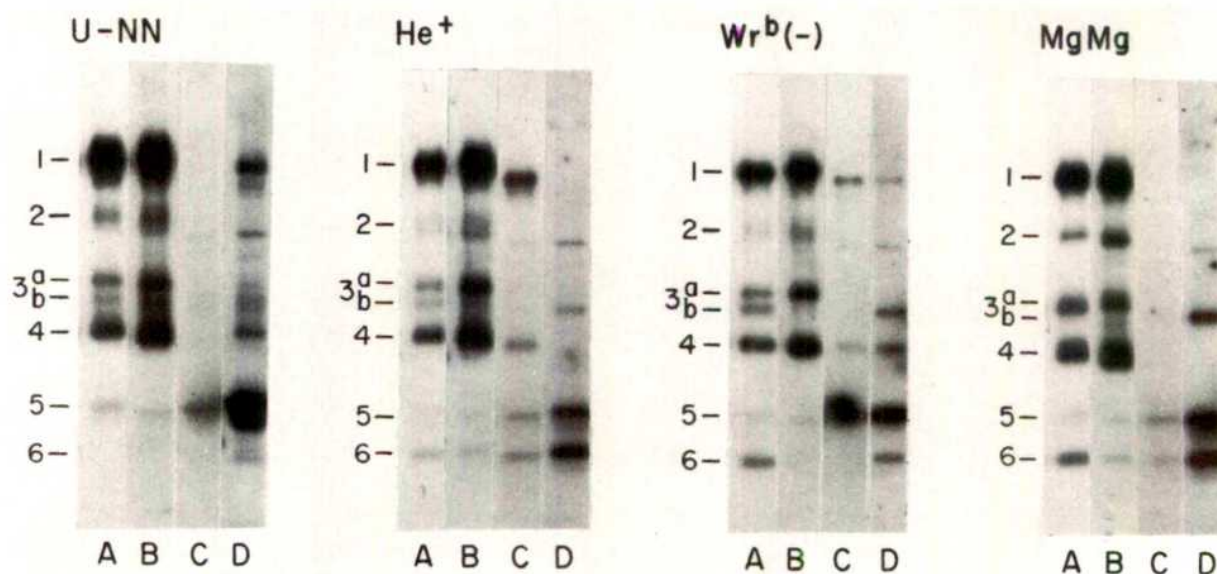


Fig 3. Fluorographs of immunoblots of U-NN, He⁺, Wrb⁽⁻⁾, and M^g erythrocytes. Lane A, treated with antiglycophorin serum; lanes B, with anti-peptide C serum; lanes C, with M MoAb; lanes D, with N MoAb.

and with the N MoAb; this supports their proposed (δ - α) hybrid structure.^{14,15,18}

The glycophorin profiles of He⁺ and Wrb⁽⁻⁾ cells are similar, under all conditions, to those of "normal" erythrocytes (Fig 3).

DISCUSSION

Using gel electrophoresis followed by immunoblotting, we demonstrate the extent of structural polymorphism of glycophorins in randomly chosen individuals, as compared to known serologic variants of the M, N blood group system.

The polyclonal antiserum to α glycophorin of an MN individual is an excellent reagent for the visualization of most molecules of the glycophorin family, and the antiserum to the carboxyl-terminal peptide of α glycophorin as well as the M and N MoAbs provide insight into the integrity of specific regions of the glycophorin molecule. In particular, this approach allowed us to identify the δ glycophorin and its variants and to detect glycophorins with altered amino terminal regions.

The presence of multiple bands in the gel patterns reflects partly the monomer-dimer relationship of α and δ glycophorins. Some bands observed in "normal" cells may represent other members of this family of proteins, for example, glycophorins in bands 3a and band 5. Glycophorin in band 3a may be a variant, and its lack of reaction with M and N MoAbs may be due to a sequence alteration or a lack of glycosylation within the amino terminal region. Reaction of the glycophorin in band 5 with both MoAbs in erythrocytes of all individuals of either M or N blood type is puzzling. Our findings could be explained by co-migration with γ glycophorin of minor glycophorins carrying M and N epitopes; no such glycophorins have been described. These glycophorins are thought to be related to α glycophorins since they all react with the antiserum to peptide C.

The mostly invariant glycophorin profiles as determined here among the 52 individuals suggest that electrophoretically definable variants are relatively infrequent. In addition, as part of another study,³¹ 54 individuals (with different pathologies) were also found to have identical glycophorin patterns, confirming that immunoblotting-definable glycophorin variants are rare. These observations complement the serologic findings of low incidence of variants of M, N, S, s blood groups.⁶ Nevertheless, in our series of 41 Africans, two individuals whose profiles are different lack δ glycophorin, and their phenotype is most likely S-s-U-. The frequency of approximately 5% observed here confirms the reported serologically defined frequency for the ethnic group we examined (Bantu-speaking Africans).³² This frequency is high enough to qualify for a selection-balanced polymorphism and because glycophorins have been implicated in *plasmodium falciparum* invasion, this relationship is worth pursuing. The structural difference in glycophorin of band 5 that was observed with the other three individuals has not been reported previously.

Our results with the serologically variant erythrocytes confirm and amplify the observations of other laboratories. This proved particularly significant in the case of hybrid glycophorins as found in Mi V or St^a cells^{13-16,18} where reaction with anti-peptide C serum or with the MoAbs could differentiate between the α - δ or δ - α hybrids and provided evidence that En(a⁻) cells used contained δ glycophorin. The use of the M and N MoAbs also confirmed the structural alteration in M^g glycophorin^{10,11} and in addition allowed us to conclude that Mi I glycophorin in band 4' was altered in the amino-terminal region and differed therefore from the analogous protein in MII cells.

The lack of an abnormal pattern in He⁺ and Wrb⁽⁻⁾ erythrocytes could be due to structural alterations of glycophorins that cannot be detected by the antisera used or to

mutations that do not alter the SDS-electrophoretic banding pattern.

Our results indicate that the erythrocytes of eight of ten MNSs blood group variants, documented serologically, show differences from the "normal" cells in electrophoretically resolved glycophorin profiles and clearly demonstrate that serologic deviation is a manifestation of structural variation in one or more members of the glycophorin family of molecules.

In a related approach, interactions with specific lectins have been used to probe variant glycophorin molecules resolved by gel electrophoresis of erythrocyte membrane proteins.³³ Merry et al used a series of MoAbs to α glycophorin to quantify the number of sites per erythrocyte.³⁴ In a

recent study Langlois et al proposed the use of flow cytometric analysis of erythrocytes labeled with specific glycophorin MoAbs to detect and analyze variant forms of glycophorins.³⁵

We conclude from this study that immunoblotting techniques, with a carefully selected battery of polyclonal and monoclonal antibodies, are capable of providing an accurate qualitative picture of polymorphism of the glycophorin system, significantly amplifying serologic analyses.

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Shear-Induced Platelet Aggregation Requires von Willebrand Factor and Platelet Membrane Glycoproteins Ib and IIb-IIIa

By D.M. Peterson, N.A. Stathopoulos, T.D. Giorgio, J.D. Hellums, and J.L. Moake

Different types of platelets in various types of plasma were subjected to levels of shear stress that produce irreversible platelet aggregation in normal platelet-rich plasma (PRP). At shear stresses of 90 or 180 dyne/cm² applied for 30 seconds or five minutes, aggregation was either absent or only transient and reversible using severe von Willebrand's disease (vWD) PRP (<1% von Willebrand factor, vWF); Bernard-Soulier syndrome (BSS) PRP (platelets deficient in the membrane glycoprotein Ib, GPIb); normal PRP plus monoclonal antibody (MoAb) to GPIb; thrombasthenic PRP (platelets deficient in membrane glycoprotein IIb-IIIa complex, GPIIb-IIIa); and normal PRP plus MoAb to GPIIb-IIIa. Shear-induced aggregation was inhibited under the above conditions, even though the platelets were activated

to release their granular contents. Sheared normal platelets in vWD plasma aggregated in response to added vWF. These studies demonstrate that the formation of stable platelet aggregates under conditions of high shear requires vWF and the availability of both GPIb and GPIIb-IIIa on platelet membranes. The experiments demonstrate that vWF-platelet interactions can occur in the absence of artificial agonists or chemical modification of vWF. They suggest a possible mechanism for platelet aggregation in stenosed or partially obstructed arterial vessels in which the platelets are subjected to relatively high levels of shear stress.

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PLATELET aggregation induced by various agonists in plasma is dependent on the binding of plasma proteins to specific ectoglycoprotein receptors on platelet surface membranes.¹ Ristocetin, a cationic glycopeptide antibiotic, artificially promotes platelet clumping in vitro by inducing the binding of large factor VIII-related von Willebrand factor (vWF) multimeric forms to or near to platelet membrane glycoprotein Ib (GPIb) molecules. Adenosine diphosphate (ADP), which is released (along with serotonin) from the dense granules of stimulated platelets, promotes platelet aggregation by inducing the attachment of fibrinogen to platelet membrane glycoprotein IIb-IIIa complexes (GPIIb-IIIa). Thrombospondin, a protein released from the α -granules of stimulated platelets, stabilizes fibrinogen binding to platelet surface glycoprotein IIb-IIIa complexes. Thrombin-stimulated platelets are altered in a manner that enables their glycoprotein IIb-IIIa complexes to bind fibrinogen, thrombospondin, fibronectin, and, with low affinity, vWF multimers.² Thus vWF is capable—under different circumstances—of binding both to glycoprotein Ib and to the glycoprotein IIb-IIIa complexes on platelet surfaces.

Patients with severe von Willebrand's disease (vWD) have an inherited bleeding disorder associated with the near absence of vWF multimers from their plasma and from their platelet α -granules.³ Von Willebrand's disease platelets have normal platelet receptors for vWF multimer binding,⁴ and platelet aggregation in severe vWD PRP is normal in response to ADP and other agonists except ristocetin. The giant platelets of patients with the congenital bleeding disorder Bernard-Soulier syndrome (BSS) lack platelet membrane GPIb¹ and cannot bind vWF or aggregate in the presence of ristocetin.⁴ Platelets in BSS PRP aggregate to ADP and other agonists. The platelets of patients with the severe form of the hereditary bleeding disorder Glanzmann's thrombasthenia lack the platelet membrane GPIIb-IIIa complex.¹ Platelets in thrombasthenic PRP aggregate in response to the ristocetin-induced binding of vWF multimers.⁴ ADP, epinephrine, and thrombin require for aggregation the binding of fibrinogen to the GPIIb-IIIa complex and, therefore, do not induce the aggregation of thrombasthenic platelets.¹

Fluid shear stress applied in vitro to normal PRP can itself induce platelets to aggregate and secrete their granular contents in the absence of added agonists.⁵⁻⁹ Shear stress may reach levels as high as 200 to 400 dyne/cm² when arterial vessels become stenosed or partially occluded,¹⁰⁻¹² as by arteriosclerosis or during vascular spasm. Thus platelet aggregation induced by shear stress may be of considerable importance both in normal hemostasis¹³ and in the pathophysiology of thrombus formation in small arteries, arterioles, and capillaries of the microcirculation.¹⁰

The present study was undertaken to evaluate the dependence of shear-induced platelet aggregation on plasma vWF and on platelet GPIb and the IIb-IIIa complex and to study their involvement in the mechanism of shear-induced platelet aggregation.

MATERIALS AND METHODS

Whole blood samples were collected from 12 healthy adults and three patients into siliconized test tubes containing 0.38% sodium citrate as anticoagulant (one volume of anticoagulant to nine volumes of blood). The patients had severe vWD (one patient; plasma and platelet vWF levels <1% of normal); BSS (one patient); and thrombasthenia (one patient).^{4,14,15} All have had life-long mucosal and posttraumatic bleeding and prolonged template bleeding times. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation as described elsewhere.⁹ The PRP

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platelet count was adjusted to 300,000/ μ L with PPP. BSS PRP had a platelet count of 50,000/ μ L; however, the BSS platelets were four times the mean volume of normal platelets.

The PRP samples were subjected to controlled shear stress levels in a stainless steel cone-plate viscometer⁹ (Ferranti Electric Model 781) at 24 °C. Shear stress-induced platelet aggregation in this instrument has been demonstrated to be independent of any minimal platelet-surface interactions.⁹ There was also no measurable attachment of vWF antigen or particular multimeric forms to the surface of the viscometer, with or without the application of shear stress, as studied by methods described elsewhere.¹⁶ The number of shear experiments with normal and patient PRP were normal PRP, 25 experiments; severe vWD PRP, four experiments; BSS PRP, four experiments; and thrombasthenic PRP, two experiments.

Some normal PRP samples were incubated for five minutes with monoclonal antibodies (MoAbs) prior to shearing in concentrations of 6 μ g/mL (6D1 antibody against GPIb; ten experiments with five normal PRP samples); 10 μ g/mL (10E5 antibody against the GPIIb-IIIa complex; six experiments with five normal PRP samples); or 20 μ g/mL (control MAB31 antibody that binds to an undefined platelet membrane antigen and produces no functional defect; four experiments with two normal PRP samples). Monoclonal antibodies to GPIb (6D1) and GPIIb-IIIa complex (10E5) were generously provided by Dr Barry S. Collier of the State University of New York at Stony Brook, and we are grateful for his important contribution. The control MAB31 MoAb was produced in our laboratory by methods described by Collier et al.^{17,18}

After shearing, 10 μ L of PRP samples were dispensed in 20 mL of Coulter Isoton II diluent containing 0.5% glutaraldehyde and were counted. Particle count and size distribution were done with an electronic particle counter (Coulter Electronics Model ZB1 with Channelyzer) using a 50 μ m diameter aperture. Particles with sizes $\pm$ 20% of the mean platelet distribution of the unsheared PRP were considered as "single platelets." It is known, of course, that this size distribution range includes some small aggregates consisting of doublets and triplets. However, studies in which EDTA was used to disaggregate control samples suggest that the "single platelets," as reported here, agree with the actual platelet numbers (EDTA-treated) to within about 30%. The disappearance of "single platelets" was accounted for by the formation of platelet aggregates; thus the percent decrease in "single platelets" is directly related to the percent increase in platelet aggregates. Lactic dehydrogenase activity (LDH) and ¹⁴C-serotonin release from platelets were measured by methods described previously.^{7,8}

Large vWF multimeric forms were purified from normal human cryoprecipitate by the method of Thorell and Blomback.¹⁴

RESULTS

The dependence of shear-induced platelet aggregation on plasma vWF and on platelet GPIb and the GPIIb-IIIa complex is shown in Figs 1 and 2. Normal platelets sheared in normal plasma at 90 or 180 dyne/cm² for varying times were aggregated extensively. Exposure to shear stress for 30 seconds or more resulted in platelet aggregation that could be observed directly and microscopically and was quantified as a decrease in the number of single platelets by nearly 80%. After five minutes of shearing some platelets disaggregated, as shown by the increase in particle count; however, about 50% remained in stable aggregates. In contrast, stable platelet aggregates failed to form in the shear fields using PRP from a patient with severe vWD; BSS (deficient in the ristocetin-induced binding component of GPIb);^{4,15} throm-

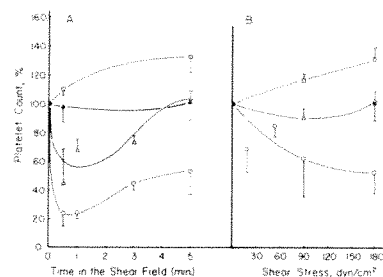


Fig 1. Effects of shear stress and shearing time on normal (○), Bernard-Soulier (□), severe von Willebrand's disease (vWD) (▲), and anti-GPIb antibody-treated (Δ) platelet-rich plasma. In (A) shear stress was 180 dyne/cm²; in (B) shearing time was five minutes. Symbols represent mean values and bars one SD.

basthenia (deficient in the GPIIb-IIIa complex);^{4,19} and normal individuals in the presence of MoAbs either to GPIb or to the GPIIb-IIIa complex.^{17,18} The shear-induced increases in particle counts observed in the BSS, thrombasthenic, and anti-GPIIb-IIIa-treated PRP was presumably due to dissolution of some microaggregates that were initially present before application of the shear field.

The control MoAb (MAB31) that binds to human platelets at a site other than GPIb or the IIb-IIIa complex did not impair shear-induced platelet aggregation in PRP, as shown in Fig 2.

PRP consisting of normal platelets in severe vWD plasma prepared by methods described elsewhere,²⁰ sheared at 180 dyne/cm² for 30 seconds or five minutes, failed to produce aggregates (data not shown). Immediately following the shearing, PRP samples placed in an aggregometer aggregated in response to added vWF (100 U/dL). This extent of aggregation was significantly higher than in nonsheared PRP samples. Sheared PRP samples, which were incubated for five minutes after the shear, aggregated in response to added vWF to a degree intermediate between the unsheared control and the sheared but not incubated specimen. Other results (not shown) revealed that vWF did not attach to the viscometer and that vWF multimeric forms were not altered in the shear field.

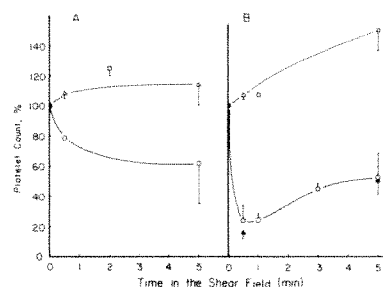


Fig 2. Effect of time in the shear field on normal (○), thrombasthenic (□), anti-GPIIb-IIIa antibody-treated (Δ) control monoclonal antiplatelet antibody (MAB31)-treated (▲) platelet-rich plasma sheared at 90 dyne/cm² (A), and 180 dyne/cm² (B). The bars denote one SD.

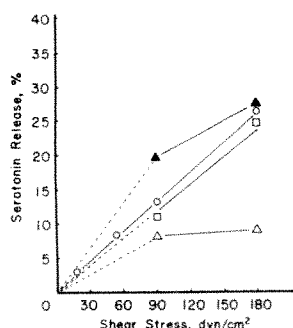


Fig. 3. Effect of shear stress on ^{14}C -serotonin release from normal (○), Bernard-Soulier (□), anti-GPIb antibody-treated (△), and anti-GPIIb-IIIa antibody-treated platelets (▲) in platelet-rich plasma sheared for five minutes.

Platelets in the different types of samples released ^{14}C -serotonin in response to shear stress (Fig 3). These results indicate that activation of the platelets occurred in the shear fields, even when there was little or no aggregation.

DISCUSSION

The present study has demonstrated that vWF and the availability of both GPIb and the GPIIb-IIIa complex on platelet membranes are essential for aggregation at relatively high shear stresses, as may be attained in stenosed or partially occluded arterial vessels.¹⁰⁻¹² No externally added agonist (eg, ristocetin) or chemical modification of vWF (eg, desialation) is required for this effect. Our findings are compatible with other observations¹³ that implied the

involvement of vWF in platelet thrombus formation in a flowing system and with a preliminary report²¹ of defective shear-induced platelet aggregation in vWD PRP. Our results may help to explain the disappearance of large vWF multimeric forms during acute episodes of systemic platelet aggregation in thrombotic thrombocytopenic purpura¹⁴ and during extensive intrarenal platelet aggregation in the hemolytic-uremic syndrome.²² Our results reported elsewhere²⁰ show that the large multimers are especially active in platelet-platelet binding in the shear field.

It is possible that vWF attachment to platelets is necessary but not sufficient to cause aggregation during the application of relatively high shear stresses. Additional plasma proteins may be involved. Our results confirm the possibility expressed by others^{23,24} that shear induces a direct alteration of platelet membrane GPIb and GPIIb-IIIa complexes. According to this hypothesis, vWF attaches predominantly to GPIb, and this binding further perturbs the conformation of GPIIb-IIIa; then additional vWF, along with fibrinogen (and, possibly, fibronectin), attach to the altered GPIIb-IIIa complexes and potentiate aggregation.

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Gamma-Interferon in Aplastic Anemia: Inability to Detect Significant Levels in Sera or Demonstrate Hematopoietic Suppressing Activity

By Beverly Torok-Storb, Gretchen Gloor Johnson, Raleigh Bowden, and Rainer Storb

A radioimmunoassay (RIA) was used to quantitate biologically active gamma interferon (INF- γ) in sera and in supernatants of cultured mononuclear cells obtained from 50 patients with aplastic anemia. Only five of the 50 serum samples had INF- γ levels above background ($>0 < 0.5$ units per mL). Detectable levels of spontaneous INF- γ (0.3 to 868 U/mL) were found in 18 of the 50 mononuclear cell supernatants tested. The addition of patient sera or INF- γ positive supernatants to cultures of normal hematopoietic colonies did not result in reduced colony growth. Flow cytometric analysis of mononuclear cells failed to

establish a correlation between the presence of INF- γ in supernatants and the number of activated T cells or natural killer (NK) cells in the mononuclear cell population. However, a significant correlation did exist between the presence of monocytes and the production of INF- γ . Contrary to previous reports, our data suggest that patients with aplastic anemia do not have high circulating levels of INF- γ . Unstimulated mononuclear cells from some patients will produce significant levels of INF- γ , but this does not result in decreased *in vitro* hematopoiesis.

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CELLS participating in a response to foreign antigen produce a cascade of lymphokines that serve to activate cellular functions required to direct and control the immune response. Among these lymphokines is a heterogeneous family of proteins known as interferons (INF). One species of INF referred to as immune or gamma INF (INF- γ) is produced by lymphocytes, particularly antigen-specific T cells and natural killer cells (NK).^{1,4} A recent review of INF- γ has summarized the multitude of effects attributed to this lymphokine.⁵ Among these effects is the inhibition of hematopoietic colonies grown *in vitro*.^{6,7} This observation, together with the detection of high levels of INF- γ in the serum of patients with aplastic anemia, has led to the hypothesis that INF- γ is the mediator of hematopoietic suppression seen in these patients.⁸

The experiments presented in this report were designed to test the role of INF- γ in hematopoietic suppression associated with aplastic anemia. The methodologies used took advantage of a recently developed solid phase radioimmune assay specific for biologically active INF- γ .⁹

MATERIALS AND METHODS

Patient samples. Included in this study were samples obtained from 50 patients with severe aplastic anemia. After informed consent whole blood was obtained by venipuncture prior to treatment. Blood was collected into preservative-free heparin, diluted 1:1 in Hanks' balanced salt solution, (HBSS) and layered over Ficoll-Hypaque for centrifugation at $400 \times G$ for 30 minutes. Interface mononuclear cells were collected, treated with hemolytic buffer, and washed three times as described previously.¹⁰ Serum samples were obtained from 3 mL clots, processed at 4 °C, and stored at -80 °C.

Interferon measurements. INF- γ was measured in a solid phase radioimmunoassay (RIA), which employs B1 antibody-coated polystyrene beads as the immunoadsorbent and ¹²⁵I-B3 antibody as a tracer (Centocor, Malvern, PA). Both the specificity of the monoclonal B1 and B3 antibodies and the sensitivity of the RIA have been described.^{9,11,12} Briefly, B1 and B3 are mouse monoclonal antibodies (MoAbs) specific for natural and *Escherichia coli*-derived recombinant human INF- γ . The two antibodies recognize different epitopes and do not compete for binding.

Two sources of recombinant INF- γ were used to test the sensitivity of the RIA. The first had a protein concentration of 1.4 mg/mL with an antiviral activity of 2.7×10^7 IU/mL, as determined by its ability to inhibit cytopathic effects (CPE) of vesicular stomatitis virus on human amnion WISH cells compared to NIH HuIFN- γ reference standard number G-023-901-527. When measured by the

RIA described above, this recombinant INF- γ had an activity of 7.78×10^6 U/mL. The second source of INF- γ had an antiviral activity of 2.0×10^6 (NIH reference) U/mL and 3.8×10^6 U/ml by RIA.

The biological activities of both sources were tested by their ability to augment NK activity against K562 tumor targets and were found to be consistent with their estimated degree of activity.¹³

Immunofluorescence labeling. Ficoll-Hypaque-separated peripheral blood mononuclear cells (PBMC) were resuspended at 1 to 3×10^6 in 0.1 mL of RPMI 1640 with 20% fetal bovine serum and then labeled as described.¹⁰ Briefly, an equal volume of MoAb at an optimal dilution was added and the cells incubated at 4 °C for 30 minutes, washed twice, and then incubated an additional 30 minutes with 0.05 mL of fluorescein isothiocyanate-conjugated goat F(ab)₂ antimouse antibody (diluted 1:10). Labeled cells were then washed, fixed in normal saline with 1% paraformaldehyde, and stored in the dark at 4 °C until analyzed.

Murine MoAbs specific for various human lymphohematopoietic surface antigens were used to label patient cells: antibodies 35.1 (CD2), 66.1 (CD4), 51.1 (CD8), all provided by Dr John Hansen, were used to identify various T cell populations; TAC antibody (a gift from Dr Tom Waldmann) was used to identify cells expressing the IL 2 receptor; antibodies 20.3 (CDw14), and 60.1 (CD11), also from John Hansen, were used to identify monocytes and granular lymphocytes, respectively; commercially available antibodies Leu-7 and Leu-11 were used to identify NK cells; antibody IF5 was provided by Dr Ed Clark for the identification of B lymphocytes.¹⁴⁻¹⁹

Cell sorter analysis. Fixed cells were analyzed by flow microfluorometry with use of a fluorescence-activated cell sorter (FACS IV, Becton Dickinson, Oxnard, CA) equipped with a five-decade

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logarithmic amplifier. Glutaraldehyde-fixed chicken red blood cells were used as standards before each analysis. For each sample the intensities of forward light scatter, 90° light scatter, and green fluorescence were determined for 6×10^3 signals. Data was stored on a floppy disc for computer analysis. The fluorescence intensity of cells labeled with specific MoAbs was compared with that of cells treated with nonbinding control antibodies to determine the percent positive cells for a given surface determinant.

Tissue culture. Mononuclear cells were cultured for one to seven days, $\pm 50 \mu\text{g/mL}$ PHA, in 10% fetal bovine serum and RPMI 1640 at a concentration of 1 to 2×10^6 cells per mL. Cells were removed from the cultures by centrifugation and the supernatants stored at -80°C until analyzed for INF- γ levels or colony-inhibiting activity.

Ficoll-Hypaque-separated bone marrow cells (BMC) or PBMC from normal donors were used as a source of hematopoietic precursor cells assayed as erythroid burst-forming units (BFU-E) and granulocytic-monocytic colony-forming units (CFU-GM). Both were grown in 0.9% methylcellulose, with 10% to 20% pooled human serum, 2% bovine serum albumin, and 3.0 U/mL of partially purified erythropoietin (Terry Fox Laboratories, Vancouver, BC). An exogenous source of burst-promoting and colony-stimulating activities was provided as supernatant from the MO-cell line (a gift from Dr D. Golde) and PHA-stimulated leukocyte-conditioned media; each was added at a final concentration of 4%.

RESULTS

Sensitivity of the RIA. Figure 1 shows the linear relationship between bound ^{125}I -B3 antibody measured as counts per minute (cpm) and units per milliliter of INF- γ added by serial twofold dilutions of a standard recombinant INF- γ source. Comparable data were obtained with two additional sources of recombinant INF- γ . The assay is linear between 1 and 50 U/mL as reported; therefore samples with greater than 50 U/mL activity were diluted for a second measurement.

Stability of INF- γ . The stability of INF- γ in fresh human serum was evaluated. The data presented in Table 1 indicate that serum samples can be held at least 24 hours at 22°C before storage at -80°C without losing activity. Nevertheless, all patient sera were processed at 4°C within six hours of procurement and then stored immediately at -80°C until analyzed. The stability of INF- γ in culture media stored at -80°C was also assessed. Four culture supernatants containing detectable levels (47, 41, 22, 14

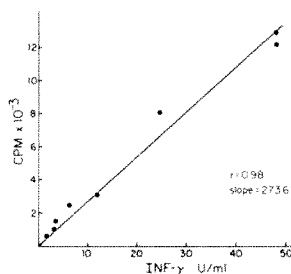


Fig 1. Standard curve showing the average counts per minute (cpm) on the vertical axis obtained for each of the INF- γ concentrations prepared as twofold serial dilutions of a recombinant INF- γ source. Background (ie, 0 $\mu\text{g/mL}$) averaged 159.5 cpm and was subtracted from all counts prior to calculation of the standard curve.

Table 1. Stability of INF- γ in Fresh Human Serum as a Function of Time Prior to Storage at -80°C

U/mL INF- γ Added	0 Minutes	Six Hours	24 Hours
2	875* (1.3)	766 (0.9)	887 (1.3)
4	1035 (1.9)	1392 (3.1)	1270 (2.7)
8	2421 (6.9)	2604 (7.6)	2512 (7.2)
40	15830 (37.1)	15020 (35.1)	14280 (33.3)

*Data represent cpm (U/mL INF- γ).

U/mL) were assayed a second time after an additional month of storage. The second measurement of all four samples (49, 37, 25, and 14, respectively) varied less than 10% from the original.

INF- γ in sera and supernatants. Forty-five of the 50 serum samples tested in this assay had no detectable amounts of INF- γ . Five samples had levels above background that were calculated to constitute less than 0.5 U/mL.

Mononuclear cells from bone marrow and peripheral blood were cultured with and without PHA for seven days. Culture supernatants were sampled daily and assayed for INF- γ . Figure 2A shows that plateau levels of INF- γ were obtained by day 3 from PHA-stimulated normal blood or marrow mononuclear cells and that no INF- γ was detected when PHA was not added to cultures. Figure 2B shows that plateau levels of INF- γ were also reached by day 3 in cultures of PHA-stimulated blood mononuclear cells obtained from four representative aplastic anemia patients. Cells from two patients produced significant levels of INF- γ without mitogen stimulation. Subsequently, supernatants were routinely harvested at day 3 from cultures of unstimulated patient peripheral blood mononuclear cells to assess spontaneous production of INF- γ . Detectable levels of INF- γ ranging from 0.3 to 868 (mean = 34.8) U/mL were observed in 18 of the 50 samples tested. Data on INF- γ production by patient marrow cells was not obtained because marrow mononuclear cells obtained from aspirates of patients either contained too few cells for culture or were so heavily contaminated with blood that they were indistinguishable by FACS analysis from PBMC.

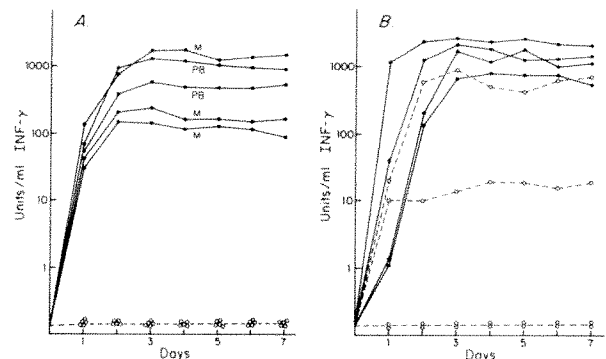


Fig 2. Production of INF- γ U/mL by mononuclear cells cultured with PHA (solid circles) and without PHA (open circles); data obtained from normal blood derived (PBMC) or marrow derived (M) mononuclear cells is shown in (A), whereas data obtained from aplastic anemia patient PBMC are shown in (B).

Table 2. Comparative Analysis of Peripheral Blood Mononuclear Cells Used to Generate Cultured Supernatants Either Positive (Group A) or Negative (Group B) for Spontaneous INF- γ Production

	Group A n = 18	Group B n = 32
INF- γ U/mL	34.8 (0.3-890)*	0
Ratio helper:suppressor T cells	1.6 (0.3-2.9)	1.7 (0.6-4.6)
% IL2 receptor positive cells	10.6 (0-27.6)	10.8 (1.3-37.4)
% Leu-7 positive cells	12.5 (0-26.9)	17.6 (3.4-47.6)
% Leu-11 positive cells	11.4 (0-38.5)	10.1 (1.0-24.5)
% CD-11 positive cells	24.1 (6.6-47.4)	25.8 (8.1-87.7)
% Monocytes†	11.2 (2.0-24.1)	<1.6 (<1.0-8.5)‡

*Data are shown as mean (range) of all observations.

†Monocytes were identified by large size, failure to label with T and B cell specific antibodies, and positive labeling with antibodies 20.3 (CDw14), 60.1 (CD11), and p4.1 (HLA-DR).

‡Twenty-three of 32 samples in group B had <1.0% detectable monocytes; for computation of the mean these samples were scored as 1.0. Therefore our statistical analysis represents a very conservative estimate of the differences between group A and group B.

Flow cytofluorometric analysis. Indirect immunofluorescence labeling and flow cytofluorimetric analysis (FACS) was used to compare the composition of PBMC between samples that produced spontaneous INF- γ and samples that did not. As shown in Table 2, PBMC that produced INF- γ (group A) had the same helper:suppressor T cell ratios, percent activated T cells, and percent HNK-1 (Leu-7) positive cells as the PBMC that did not produce INF- γ (group B). In contrast to these similarities, the PBMC from group A had a significantly ($P < 0.001$) greater percentage of monocytes than found in group B. The majority of the PBMC populations in group B (23 out of 32) had less than 1% monocytes as determined by FACS analysis of 6×10^3 total cells.

INF- γ effects on hematopoietic colony growth. Supernatants from cultured PBMC were added at a final concentration of 10% to in vitro assays of hematopoietic precursors. Table 3 summarizes the data obtained in one representative experiment, which indicates that INF- γ positive supernatants had no significant effect on BFU-E or CFU-GM growth. All 18 INF- γ positive supernatants were tested in cultures of normal blood derived BFU-E. BFU-E numbers obtained in cultures with INF- γ positive supernatants ranged from 90% to 120% (mean 101.5) of growth obtained in control cultures without supernatants. Ten supernatants with no detectable INF- γ were also tested; BFU-E growth in the presence of negative supernatants ranged from 46% to 160% (mean = 94.3) of control growth.

Table 3. Representative Experiment Showing the Effect of Supernatants From Cultured Mononuclear Cells on the Growth of Normal Erythroid Burst Forming Units (BFU-E) and Granulocytic/Monocytic Colonies (CFU-GM)

Supernatants Added*	Mean $\pm$ SE colonies/ 10^5 Cells	
	BFU-E	CFU-GM
None	61 $\pm$ 0.7	24 $\pm$ 3.2
Normal (0 U/mL INF- γ)	52 $\pm$ 6.4	25 $\pm$ 2.7
Patient 1 (868 U/mL INF- γ)	58 $\pm$ 3.5	23 $\pm$ 1.8
Patient 2 (0 U/mL INF- γ)	64 $\pm$ 9.1	21 $\pm$ 3.3
Patient 3 (83.6 U/mL INF- γ)	51 $\pm$ 4.9	19 $\pm$ 2.7

*All supernatants were harvested from cultures of unstimulated PBMC at day 3 of incubation.

The effect of 10% patient serum on the growth of normal BFU-E was also evaluated. None of the 20 samples studied contained growth inhibiting activity (data not shown).

DISCUSSION

Severe aplastic anemia most likely results from several different pathogenic mechanisms.²⁰ Clinical data showing recovery in a proportion of patients after cytoreductive or immunosuppressive therapy strongly suggest that in some cases aplasia must be caused by mechanisms extrinsic to the stem cell.²¹⁻³⁰ Investigators have focused on activated T cells or NK cells and their products as the possible effectors of this mechanism with the stem cell or the putative hematopoietic regulatory cell as the target.³¹⁻⁴² Recently particular emphasis has been placed on INF- γ elaborated by activated T lymphocytes as the direct mediator of hematopoietic suppression.^{6-8,34,37-39}

Implicating INF- γ as a direct effector of hematopoietic suppression in vitro has primarily involved studies that used partially purified sources of INF- γ . Recent findings, however, suggest that even the most purified preparations of INF- γ have been contaminated with lymphotoxins that may be responsible for growth inhibition.⁴³ Recently Raefsky et al reported that colony growth could be inhibited with a homogenous preparation of recombinant INF- γ ; however, the extent of inhibition was influenced by culture conditions such that maximum inhibition required suboptimal concentrations of fetal bovine serum.⁴⁴ In contrast to this, Trinchieri and Perussia have reported studies that showed that even high doses of recombinant INF- γ did not significantly inhibit colony formation.⁵

The association of INF- γ with aplastic anemia came from the observations that relatively high levels of INF- γ could be detected in patient serum and in supernatants from cultured patient cells.⁸ The presence of INF- γ was determined in those studies by its ability to inhibit the cytopathic effects of vesicular stomatitis virus on human amnion WISH cells. The presence of INF- γ in serum of aplastic anemia patients seemed reasonable, since their peripheral blood mononuclear cells also contained an elevated number of activated lymphocytes that are reported capable of producing INF- γ . However, using a very sensitive and reproducible RIA specific for

biologically active INF- γ , we were unable to detect elevated levels of INF- γ in the serum of aplastic anemia patients.

The difference between our serum results and those previously reported could possibly be attributed either to a qualitative difference between patient groups or the possibility that the bioassay of virus inhibition may be detecting several activities, whereas the RIA is only detecting INF- γ . Ideally, the same serum samples should be evaluated in both assays. Recently Hinterberger et al, using both the standard bioassay and the Centocor RIA, reported that INF- γ was not detected in the serum of patients with aplastic anemia.⁴⁵

We also measured the release of INF- γ from cultured peripheral blood mononuclear cells (PBMC). PHA stimulated PBMC from both patients, and normals produced significant amounts of INF- γ , which reached plateau levels by day 3 of incubation. Unlike normal PBMC, cells from some patients released significant levels of INF- γ without PHA stimulation as previously reported.⁸ This spontaneous release also reached plateau levels at day 3. Therefore, all patients were subsequently tested for spontaneous INF- γ production at day 3 of culture; 18 of the 50 samples tested had detectable levels. All 18 supernatants were tested for their ability to inhibit growth of hematopoietic colonies, and none showed significant inhibition. As a group the INF- γ positive supernatants had the same effect on BFU-E growth as INF- γ negative supernatants. Sera from these patients had no inhibitory effect on normal BFU-E growth. These

observations are in agreement with those obtained by David Nathan and colleagues who studied a series of 15 patients and failed to demonstrate any inhibitory activity in patient sera (personal communication).

The ability of the cultured PBMC to produce INF- γ did not correlate with the presence of activated T lymphocytes or HNK-1 positive cells; however, INF- γ production was significantly ($P < 0.001$) correlated with the presence of greater than 1% monocytes.

Since INF- γ production by T lymphocytes and NK cells has been shown to be dependent upon the secretion of interleukins by accessory cells,⁴⁶ it is not surprising that we found an association between the absence of monocytes in patient PBMC and the inability of the PBMC to produce INF- γ . We cannot be certain at this time whether the relative paucity of monocytes recovered from the majority of patients reflects problems associated with cell separation and recovery or the real consequences of a disease process. If our assessment of monocyte number accurately reflects the in vivo situation, it becomes reasonable to hypothesize that insufficient numbers of monocytes could contribute to immune dysfunction, and given that the immune system may serve in some capacity to regulate hematopoiesis, such immune dysfunction could serve to maintain marrow failure. The data presented in this report, however, do not support the hypothesis that INF- γ is the direct effector of "immune-mediated" aplasia.

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Inosine Monophosphate Dehydrogenase and Myeloid Cell Maturation

By Robert D. Knight, John Mangum, Diane L. Lucas, David A. Cooney, Ernest C. Khan, and Daniel G. Wright

In previous studies of purine ribonucleotide metabolism in the human myeloid leukemia cell line HL-60, we observed that there is a down-regulation of guanine ribonucleotide biosynthesis from the central intermediate, inosine monophosphate (IMP) and a depletion of intracellular guanosine triphosphate (GTP) and guanosine diphosphate (GDP) pools that occur during the induced maturation of these cells. We also found that inhibitors of IMP dehydrogenase, the enzyme that catalyzes the first step of guanylate synthesis from IMP, are potent inducers of HL-60 maturation. Because of these observations we specifically investigated the activity of IMP dehydrogenase in HL-60 cells and in a new inducible human myeloid leukemia cell line, RDFS2-25, both during maintenance culture and during induced maturation of the cells. Enzyme activity was examined directly in cell extracts with a radiometric assay that measures free $^3\text{H}_2\text{O}$ formed from $[2\text{-}^3\text{H}]$ IMP during the conversion of IMP to XMP. Uninduced HL-60 and RDFS2 cells in maintenance culture were found to have high levels

of IMPD activity (5.2 to 5.7 pmol IMP metabolized/ 10^7 cells/min) compared with normal neutrophils and monocytes that had been purified from blood (<1.5 pmol IMP metabolized/ 10^7 cells/min). However, when HL-60 and RDFS2-25 cells were induced to mature with retinoic acid (10^{-6} mol/L), dimethylformamide (6×10^{-2} mol/L), or a known IMPD inhibitor, tiazofurin (10^{-6} mol/L), IMPD activity in the cells fell by 51% to 80% within three to six hours. These changes in IMPD activity preceded detectable functional and antigenic maturation of the cells by at least 12 hours and were not temporally related to changes in cellular proliferation. These findings are consistent with the concept that the regulation of myeloid cell maturation may be influenced by intracellular concentrations of guanine ribonucleotides because IMP dehydrogenase activity is known to be rate limiting for the production of these nucleotides.

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THE NEOPLASTIC cells of acute myelogenous leukemia are characterized by an inability to undergo normal terminal differentiation. However, defects of differentiation in leukemic cells are not necessarily complete. Leukemic cells may display variable degrees of maturity in individual patients,¹ and they may retain a capacity for spontaneous maturation when placed in primary tissue culture.² Furthermore, a number of continuously maintained, myeloid leukemia cell lines have been established that undergo maturation when exposed to a variety of chemical agents in vitro.³ Understandably these cell lines have attracted considerable investigative interest since they appear to provide an opportunity to define basic mechanisms underlying the blockade of terminal differentiation in leukemia.

The HL-60 cell line, originally isolated from the leukemic blasts of a patient with acute promyelocytic leukemia,⁴ has been the object of particularly extensive investigation. This cell line can be induced experimentally to undergo a form of terminal differentiation with which the cells acquire functional and morphological characteristics of mature neutrophils.⁵⁻⁷ In previous studies of HL-60 cells we showed that discrete changes in the intracellular production and supplies of guanine ribonucleotides guanosine triphosphate [GTP] and guanosine diphosphate [GDP] are associated with

induced maturation of these cells.⁸ Intracellular concentrations of guanine ribonucleotides were found to fall during HL-60 cell maturation, and this change could be attributed to diminished nucleotide biosynthesis. Furthermore, changes in guanylate production during induced maturation were not accompanied by comparable changes in adenylate production or in intracellular adenosine nucleotide concentrations.

Studies of purine nucleotide biosynthesis using radiolabeled precursors to examine both the de novo and purine salvage pathways during HL-60 cell maturation focused our attention upon the first step of guanylate biosynthesis from the central intermediate inosine monophosphate (IMP), catalyzed by the enzyme IMP dehydrogenase (IMPD). This step appeared to be the point at which guanylate biosynthesis is selectively down-regulated during induced maturation of these cells. We therefore examined the effects of known inhibitors of IMPD upon HL-60 cells and found that these agents (eg, mycophenolic acid, 3-deazaguanosine, and 2- β -D-ribofuranosylthiazole-4-carboxamide [tiazofurin]) not only selectively inhibit guanylate biosynthesis in the cells, as expected, but also induce the cells to mature.^{8,9}

These findings were of particular interest because of previous reports by others suggesting that IMP dehydrogenase activity in cells may be relevant to neoplastic transformation and/or to cellular maturity. Rat hepatoma cells^{10,11} and human leukemic blasts¹² have been found to express high levels of IMPD activity when compared to normal, differentiated cells of the same tissues. Moreover, such differences in IMPD activity, which distinguish normal from neoplastic cells, could not be explained simply by differences in cellular proliferation.¹¹

In the studies reported here, we used a radiometric assay of IMPD to study the activity of this enzyme directly in HL-60 cells and in a new human myeloid leukemia cell line, RDFS2-25.¹³

Cells were studied both in maintenance culture and during induced maturation. Results confirmed our impression that a down-regulation of IMPD occurs early during the induced

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maturation of myeloid leukemia cells, and they lend support to the concept that the intracellular supplies of guanine ribonucleotides, which are determined by the activity of this rate-limiting enzyme for guanylate synthesis, may have a role in the regulation of myeloid cell maturation.

MATERIALS AND METHODS

Human myeloid leukemia cell lines. Continuous suspension cultures of the HL-60 cell line were established initially from seed cultures obtained from the laboratory of Dr Robert C. Gallo (National Cancer Institute, Bethesda, MD). Additional stocks cryopreserved at passage 11 following the original establishment of this line, were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 medium supplemented either with 10% heat-inactivated (56 °C for 30 minutes) fetal bovine serum (Reheis Chemical, Phoenix, AZ) or with insulin (6 μ g/mL), transferrin (5 μ g/mL), and selenium (3 nmol/L) (Sigma, St. Louis). In our studies serum-free medium supplemented with insulin, transferrin, and selenium is referred to as ITS medium. Cells were cultured in 75-cm² tissue culture flasks (Falcon Plastics, Becton Dickinson, Oxnard, CA) and incubated at 37 °C in a continuously maintained environment with 7% CO₂ and 100% humidity. Cell counts in culture specimens were determined by hemocytometer chamber counting, and the viability of cells was assessed by dye exclusion using 0.1% trypan blue (MA Bioproducts, Walkersville, MD). Morphology of cells was examined with cytospin preparations stained by Wright-Giemsa. For maintenance cultures, cells were passed by resuspending them in fresh nutrient media at a concentration of 2×10^5 cells/mL every three to five days. Cells were studied at maintenance passages 20 to 30.

The RD62 cell line was isolated from the peripheral blood blasts of a 56-year-old man with acute myelogenous leukemia (FAB class M1).¹³ Shortly after diagnosis the patient died from a cerebral hemorrhage and was found to have diffuse leukemic infiltration of the brain, lymph nodes, and central organs. Leukocytes were obtained for culture at the time of diagnosis when the WBC count was 188,000/ μ L (71% blasts, 5% promyelocytes or myelocytes, and 2% lymphocytes). Leukemic blasts were peroxidase positive and α -naphthol butyrate esterase negative. These cells were separated from blood by Hypaque-Ficoll gradient centrifugation and then cultured in RPMI 1640 supplemented with 20% fetal bovine serum (Reheis), ITS, and 10% PHA-stimulated, human lymphocyte-conditioned media (LCM) at a cell concentration of 1×10^6 /mL. After three weeks initial culture at 37 °C with 7% CO₂, additional medium was added and then renewed weekly by demidepletion with gradual withdrawal of the fetal bovine serum (FBS) and LCM. By eight weeks cells were continuously maintained (average doubling time = 56 hours) in RPMI, ITS, and 10% FBS. These cells showed a limited but stable capacity to undergo induced myeloid maturation. This initial RD62 line was then cloned by limiting dilution, and derivative lines were isolated, which demonstrated either limited degrees of induced maturation or prominent myeloid maturation when exposed to inducing agents. Two such derivative lines, RD62-25 (inducible) and RD62-4 (noninducible), were examined in the studies reported here. Like the HL-60 cells, RD62 cells were resuspended in fresh nutrient media every three to five days. These cells were studied at maintenance passages 20 to 40.

Induced maturation of HL-60 and RD62 cells. Before IMPD levels were studied in leukemic cell lines during induced maturation, the cells were adapted to and maintained in serum-free ITS media for at least three passages. Cells in log-phase growth (two to three days following media change) were induced to mature by inoculation into fresh ITS media supplemented with a variety of inducing compounds. Final concentrations of inducing agents were retinoic

acid (Sigma), 1.0 μ mol/L; dimethylformamide (Eastman Kodak, Rochester, NY), 60 mmol/L; and 2- β -D-ribofuranosylthiazole-4-carboxamide (or tiazofurin, obtained from the Drug Synthesis and Chemistry Branch, DCT, NCI, NIH, Bethesda, MD), 1.0 μ mol/L. Induced maturation of the cells was assessed by previously described methods for determining NBT reduction,^{8,14} phagocytosis of opsonized yeast,⁸ and reactivity to the monoclonal antibody (MoAb) OKM1 (Ortho Diagnostics, Raritan, NJ) detected by FACS analysis.¹⁵

For certain studies HL-60 cells were incubated for four days with and without 5 mmol/L α -difluoromethyl ornithine (DFMO) (Merrill Dow, Cincinnati), which arrests cellular proliferation but does not induce maturation.¹⁶ For these studies cells were placed into fresh ITS media with and without DFMO every 24 hours as described previously.¹⁶

Measurement of IMP dehydrogenase in cell extracts. Cell pellets (containing 5×10^6 to 5×10^7 cells) were disrupted by sonication in 1.0 mL Tris buffer (0.1 mol/L Tris-HCl, 0.02 mol/L KCl, 1.0 mmol/L dithiothreitol, pH 8.1) over ice. Sonicates were then centrifuged at $12,000 \times g$, and IMPD was extracted from the supernatant by acid precipitation with 0.5 mL of 1.0 mol/L sodium acetate, pH 5.0, added to 1.0 mL of supernatant. After a two-minute repeat centrifugation at $12,000 \times g$, the pelleted precipitate was redissolved in 500 μ L Tris buffer, pH 8.1. Insoluble materials were eliminated by recentrifugation and the supernatant extract used as an enzyme source. Total protein concentrations in cell sonicates and in cell extracts were determined by the Bio-Rad colorimetric method (Bio-Rad Laboratories, Richmond, CA).

IMPD activity in cellular extracts was then measured by a radiometric assay, modified from methods previously reported by Cooney et al.¹⁷ This assay is based upon the recovery of tritiated water produced during the IMPD-mediated generation of XMP from [2-³H] IMP. For the assay 100- μ L aliquots of cell extracts were incubated at 37 °C with 50 μ L of allopurinol (20 μ mol/L); 50 μ L of 0.1 mol/L uridine (Sigma), and 5 μ L of [2-³H] IMP [200 μ Ci/mL; 225 nmol/mL; prepared from [2-³H] hypoxanthine as described previously¹⁷] with NAD (Sigma; final concentration = 70 μ mol/L) in a total reaction volume of 205 μ L. The IMPD reaction was terminated after incubation at 23 °C for zero to 120 minutes by applying 100- μ L aliquots of the reaction mixtures to replicate 6 $\times$ 30 mm Dowex-1-C1⁻ columns (Bio-Rad), followed by elution of the columns with 1.9 mL distilled H₂O and collection of eluants in scintillation vials with 20 mL Hydrofluor (National Diagnostics, Summerville, NJ). In this assay, anion exchange resin columns, which absorb unreacted [2-³H] IMP while ³H₂O is eluted, were used instead of saturated KOH solution as originally described¹⁷ to separate the reaction products. This modification improved the reproducibility and efficiency of the assay in our hands; nonetheless, results were similar with both versions of the radiometric assay. The radioactivity of released tritium was measured in a beta-scintillation counter and converted to pmol of IMP metabolized per minute. Enzyme activity was expressed both in relationship to the number of cells extracted and to total extracted protein added to the reaction mixture.

The specificity of the dehydrogenation reaction of IMP to XMP was confirmed by reverse phase and ion exchange chromatography,⁸ and by comparing reaction conditions with and without added NAD. Breakdown of the radiolabeled IMP to inosine by nonspecific phosphatase activity was minimal (5% of total radioactivity) to undetectable.

Measurement of intracellular purine ribonucleotide concentrations. Purine ribonucleotides in perchlorate extracts of 2×10^7 cell pellets were analyzed by reverse phase HPLC using an anion exchange, microparticulate column (Partasil-10 SAX; Whatman, Clifton, NJ) as described previously.⁸ Ribonucleotide (GTP and

ATP) concentrations (nmol/ 10^7 cells) were derived from chromatographic peaks detected by UV absorbance at 254 nm, which were related to standards chromatographed at known concentrations.

Purification of normal peripheral blood neutrophils and monocytes. Neutrophils and monocytes were separated from venous blood samples (50 to 100 mL) obtained from normal volunteers. Neutrophils (>95% purity) were isolated by dextran sedimentation of whole blood followed by centrifugation on Hypaque-Ficoll density gradients (Ficoll-Paque; Pharmacia, Piscataway, NJ) and hypotonic lysis of residual erythrocytes.¹⁸ Blood monocytes (>90% purity) were isolated from the buoyant mononuclear cells, recovered from Hypaque-Ficoll gradient-separated blood, by counter-flow elutriation as described previously.¹⁹

Presentation and statistical analysis of results. Unless otherwise noted, results are expressed as mean values $\pm$ the standard error of the mean for replicate measurements. *P* values for the statistical significance of differences were determined by the Student's *t* test.

RESULTS

IMP dehydrogenase activity in human myeloid leukemia cells lines and in normal blood leukocytes. IMPD activity was readily detected in extracts of HL-60 cells harvested from maintenance culture. Dehydrogenation of IMP by extracts of 2×10^7 HL-60 cells was linear during the first 20 minutes of incubation with radiolabeled substrate but slowed thereafter. Therefore attention was given to using incubation times and sample dilutions that would conform to linear reaction conditions. Under such conditions IMPD activity was directly related both to the numbers of cells from which extracts were prepared (within a range of 1.0 to 3.0×10^7 cells) and to the protein content of cell extracts (within a range of 0.2 to 3.0 mg cell protein).

Consistent with previous reports that leukemic blast cells have higher levels of IMPD than do mature leukocytes,¹² both HL-60 and RFD2-25 cells were found to have substantially higher levels of IMPD activity than did purified, normal blood neutrophils or monocytes. Levels of IMPD activity expressed as pmol IMP metabolized/ 10^7 cells/minute ($\pm$ SEM, $n = 6$) were 5.7 ± 0.5 and 5.2 ± 0.2 for HL-60 and RFD2-25 cells respectively, compared with 1.1 ± 0.4 and 1.4 ± 0.4 for neutrophils and monocytes; IMPD levels expressed as pmol IMP metabolized/mg cell protein/min ($\pm$ SEM, $n = 6$) were 27.6 ± 2.2 and 20.6 ± 0.8 for HL-60 and RFD2-25 cells, compared with 4.5 ± 1.7 and 7.5 ± 2.3 for neutrophils and monocytes.

IMP-dehydrogenase activity in myeloid leukemia cells during induced maturation. Substantial decreases in IMPD activity were observed in HL-60 cells by three hours of incubation with agents that induced maturation of the cells (Figs 1A and 1B); these decreases in IMPD activity were apparent both when enzyme activity was expressed in relationship to cell numbers and when expressed in relationship to milligrams of extracted protein assayed. The most rapid decline in IMPD activity was observed in cells treated with tiazofurin; in these cells IMPD levels were decreased by more than 75% within six hours and remained low thereafter (The slight rise in IMPD levels between six and 24 hours apparent in Figs 1A and 1B is not statistically significant). The decline in IMPD activity in cells treated with retinoic acid and dimethylformamide was somewhat more gradual

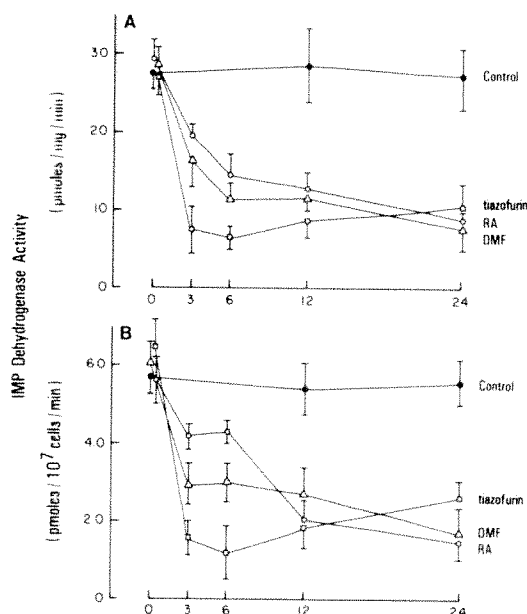


Fig 1. Rapidly declining IMP dehydrogenase activity in HL-60 cells during incubation with inducing agents. IMPD activity is expressed both in terms of milligrams of cell protein extracted (A) and in terms of number of cells extracted (B). Results represent mean values $\pm$ SEM from six replicate studies. Solid circles: control cells not exposed to inducing agents; open circles: cells incubated with retinoic acid (10^{-6} mol/L); open triangles: cells incubated with dimethylformamide (60 mmol/L); open squares: cells incubated with tiazofurin (10^{-6} mol/L).

than in tiazofurin-treated cells but was fully established by 12 hours of incubation with the inducing agents. In all cases substantially decreased IMPD levels were detectable at least 12 to 18 hours before cellular maturation was apparent either by measurement of phorbol diester-stimulated NBT reduction, by measurement of the myeloid surface antigen OKM1 (Figs 2B and 2C), or by measurement of phagocytic activity (data not shown).

Changes in IMPD levels during induced maturation of HL-60 cells were not related temporally to changes in cellular proliferation. IMPD levels fell markedly during the first 12 hours of induction (Figs 1A and 1B) before significant changes in the cell growth rate ($P < 0.05$) were observed (Fig 2A). Furthermore, DFMO, which markedly inhibits HL-60 cell growth but does not induce maturation,¹⁶ was not found to cause either decreased IMPD levels in the cells or changes in intracellular guanine ribonucleotide concentrations, unlike the maturation inducers, retinoic acid and dimethylformamide (Table 1).

Decreased IMPD levels in association with induced myeloid maturation were also observed in another human leukemia cell line, RFD2-25. This line was isolated from a patient with acute myelogenous leukemia, FAB class M1 (see "Materials and Methods" section) and is unrelated to the HL-60 line. As in HL-60 cells, IMPD activity in RFD2-25 cells was decreased by more than 50% within six hours of incubation with retinoic acid, dimethylformamide, or tiazofurin and remained at these diminished levels during

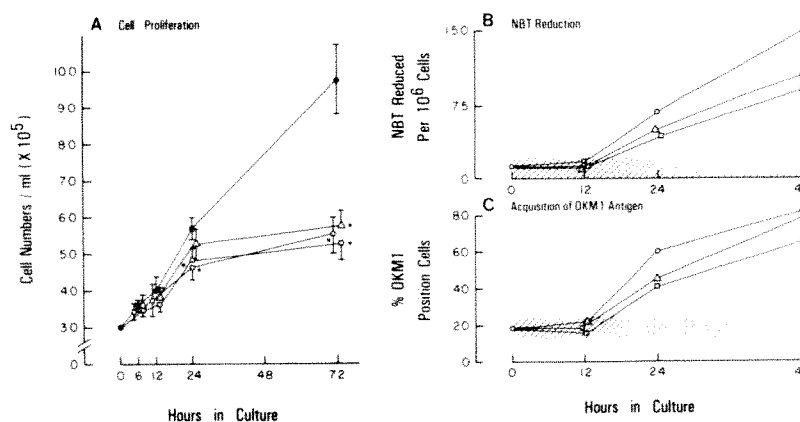


Fig 2. (A) Increase in cell numbers during 72-hour culture for HL-60 cells incubated without inducing agents (closed circles), with retinoic acid, 10^{-6} mol/L (open circles), with dimethyl-formamide, 60 mmol/L (open triangles), or with tiazofurin, 10^{-6} mol/L (open squares). Results represent mean values $\pm$ SEM from four replicate studies; data points marked with an asterisk (*) are significantly different from control data for cells incubated without inducing agents ($P < 0.05$). (B and C) Acquisition of functional maturity by HL-60 cells incubated with inducing agents: (B) NBT reduction by 10^6 cells stimulated with phorbol myristate acetate (100 ng/mL) for 30 minutes (NBT reduction is expressed as Δ OD units₅₁₅/30 minutes); (C) Percent of cells expressing the OKM1 antigen. Cross-hatched horizontal bars represent limits of mean values $\pm$ 2 SD from six separate studies for control, uninduced cells examined at 0, 12, 24, and 48 hours of incubation. Open circles: cells incubated with retinoic acid (10^{-6} mol/L); open triangles: cells incubated with dimethylformamide (60 mmol/L); open squares: cells incubated with tiazofurin (10^{-6} mol/L). Results are mean values from triplicate measurements.

subsequent culture. A separate strain of RDFS2, RDFS2-4, which was segregated from the parent RDFS2 isolate following the eighth passage of this line by a limiting dilution cloning technique, was found to be resistant to maturation induction. These cells showed no decrease in IMPD activity in response to retinoic acid or dimethylformamide (Table 2), which were also ineffective in inducing these cells to mature. RDFS2-4 cells showed only a modest decline in IMPD activity ($<30\%$) when incubated with the IMPD inhibitor, tiazofurin (10^{-6} mmol/L), which was correspondingly relatively ineffective in inducing maturation, although a modest degree of induced maturation could be detected (Table 2).

DISCUSSION

Inosine monophosphate dehydrogenase (IMPD) catalyzes the first step unique to guanylate biosynthesis from the

central purine intermediate, IMP, whereby IMP is converted to XMP. This irreversible reaction is known to be subject to both negative and positive feedback controls and to be rate limiting for the formation of guanine ribonucleotides in cells under physiologic conditions.^{20,21} We investigated the activity of this enzyme in two human myeloid leukemia cell lines, HL-60 and RDFS2-25, and in normal peripheral blood neutrophils and monocytes using a radiometric assay. We found both myeloid cell lines to have substantially higher levels of IMPD than purified normal blood neutrophils or monocytes. In addition, we observed that induced maturation of the leukemic cell lines is associated with a reduction in the activity of IMPD toward those levels observed in normal, terminally differentiated neutrophils and monocytes. Moreover, decreased IMPD activity was found to be a very early feature of induced maturation. A significant decline in

Table 1. IMP Dehydrogenase Levels in HL-60 Cells With and Without Induced Maturation

Incubation Conditions	IMPD Activity*	Intracellular [GTP] (nmol/ 10^7 cells)†	Cellular Proliferation (Percent increase in cell concentration by four days)‡	Myeloid Maturation (NBT reduction by cells after four days culture)§
Control	6.3 $\pm$ 0.5	12.3 $\pm$ 1.1	326 $\pm$ 40	12 $\pm$ 2
Retinoic acid (10^{-6} mol/L)	1.8 $\pm$ 0.3	6.8 $\pm$ 0.7	74 $\pm$ 23	173 $\pm$ 12
Dimethylformamide (60 mmol/L)	1.7 $\pm$ 0.4	5.4 $\pm$ 0.5	95 $\pm$ 30	115 $\pm$ 11
DFMO (5mmol/L)	7.7 $\pm$ 0.3	12.7 $\pm$ 1.5	53 $\pm$ 27	7 $\pm$ 1

*IMPD activity in cells after 24-hour incubation with or without inducing agents, expressed as pmol IMP metabolized/ 10^7 cells/min (mean $\pm$ SEM; n = 6).

†GTP concentrations measured in perchlorate extracts of cell pellets prepared after 24-hour incubation of cells with or without inducing agents⁸; results represent means $\pm$ SEM from four replicate studies.

‡Measurements of cellular proliferation represent the percent increases in cell concentrations during four days' culture from an initial seeding concentration of 3×10^5 cells/mL [percent increase = absolute increase in cell concentration ($\times 10^5$ /mL)/ 3.0×10^5]; results represent means $\pm$ SEM; n = 4.

§NBT reduced by 10^6 cells stimulated with phorbol diester (Δ OD units₅₁₅/30 min); mean $\pm$ SEM, n = 4.

||Control cells not exposed to inducing agents.

Table 2. IMP Dehydrogenase Activity, Intracellular GTP Concentrations, and Induced Myeloid Maturation in RD62-25 and RD62-4 Cells

	RD62-25				RD62-4			
	Control	RA (10^{-6} mol/L)	DMF (60 mmol/L)	tiazofurin (10^{-6} mol/L)	Control	RA (10^{-6} mol/L)	DMF (60 mmol/L)	tiazofurin (10^{-6} mol/L)
IMPD activity*	5.2 ± 0.2	2.3 ± 0.1	3.0 ± 0.4	1.6 ± 0.5	5.3 ± 0.7	5.5 ± 0.6	5.5 ± 0.6	3.9 ± 0.9
Intracellular [GTP]†	11.8 ± 1.2	5.7 ± 0.3	5.3 ± 0.5	4.4 ± 0.7	13.2 ± 1.5	12.5 ± 0.7	14.1 ± 2.0	9.1 ± 1.1
NBT reduction‡	11 ± 6	92 ± 5	84 ± 6	75 ± 6	12 ± 4	17 ± 5	13 ± 4	26 ± 2
Phagocytosis§	14 ± 4	78 ± 6	86 ± 9	72 ± 11	11 ± 3	12 ± 5	15 ± 4	24 ± 3

*IMPD activity in cells after 24-hour incubation with or without inducing agents, expressed as pmol IMP metabolized/ 10^7 cells/min (mean values ± SEM; n = 6).

†GTP concentration (nmol/ 10^7 cells) measured in perchlorate extracts of cell pellets prepared after 24-hour incubation of cells with or without inducing agents⁸; mean values ± SEM; n = 4.

‡Percent of cells reducing NBT when stimulated by phorbol diester⁸ after four days' incubation with and without inducing agents (mean values ± SEM; n = 4).

§Percent of cells capable of ingesting opsonized yeast⁸ after four days' incubation with and without inducing agents (mean values ± SEM; n = 4).

IMPD activity was evident by three to six hours of incubation of HL-60 and RD62-25 cells with inducing agents and preceded the functional and antigenic maturation of these cells by at least 12 to 18 hours.

Diminished IMPD activity in HL-60 and RD62 cells treated with tiazofurin was not anticipated, since this agent is known to inhibit IMPD directly and selectively.^{22,23} However, the mechanism by which IMPD is down-regulated in these cells when exposed to retinoic acid or dimethylformamide is unclear. Nonetheless, these findings are consistent with our previously reported observation that guanine ribonucleotide biosynthesis from IMP is reduced in HL-60 cells during induced maturation in part because of an apparent block at the IMPD-mediated step.⁸

Several additional observations in our present studies lend further weight to the conclusion that reduced levels of IMPD activity and myeloid cell maturation are related. Decreased IMPD levels were not observed in a strain of RD62 cells that is resistant to maturation-induction when these cells (RD62-4) were incubated with the inducing agents retinoic acid and dimethylformamide. Moreover, changes in IMPD activity observed with cellular maturation could be dissociated from altered rates of cell division. Not only did IMPD levels fall in the myeloid cell lines before the decline in cell growth rate that accompanies induced maturation, but IMPD levels did not fall in cells exposed to DFMO, which blocks cellular proliferation but does not induce maturation.¹⁶

Becker and Lohr¹² have reported previously that the circulating blasts of patients with acute myelogenous leukemia have high levels of IMPD activity compared with those of normal blood leukocytes recovered from healthy subjects. Although our methods for measuring IMPD differ from those used in these earlier studies, our results with HL-60 and RD62 cells are quantitatively similar to those reported for freshly isolated leukemic blast cells.¹²

A relationship between the activity of IMPD and neoplastic transformation has been suggested by Jackson et al.¹⁰ These investigators found that rat hepatoma cells had substantially higher levels of IMPD activity than did normal liver cells. IMPD activity in hepatoma cells was also greater than that in normal regenerating liver tissue with similar cell

growth rates.^{10,11} Although we were unable to compare the IMPD levels of leukemic cells with those of a comparably homogenous preparation of normal myeloid progenitor cells, our studies support the idea that a high level of IMPD activity in cells is a biochemical marker of cellular immaturity. Not only do HL-60 and RD62 cells have high levels of IMPD compared with their normal, terminally differentiated blood cell counterparts, but they also express diminished IMPD activity in association with induced maturation, whereby the block in terminal differentiation characteristic of these neoplastic cells in maintenance culture is circumvented or partially removed. At the same time it is important to point out that the induction conditions examined in our studies are among those that promote a form of cellular maturation reminiscent of neutrophil differentiation.⁵⁻⁷ We did not examine induction conditions associated with cellular maturation to macrophage-like^{24,25} or eosinophil-like cells,²⁶ and our results may not necessarily apply to such conditions.

While diminished IMPD activity may be simply a biochemical consequence of cellular maturation, there is reason to believe that the activity of this enzyme may have a role in regulating the maturation process by controlling intracellular supplies of guanine ribonucleotides. Several lines of evidence support this latter possibility. First, we have shown previously that IMPD inhibitors are potent inducers of maturation in human myeloid cell lines.^{8,9} Second, we have shown in more recent studies²⁷ that the maturation-inducing effects of IMPD inhibitors on HL-60 cells can be blocked by preventing the depletion of guanine ribonucleotide pools that ordinarily results from IMPD inhibition; this is accomplished by incubating cells with guanosine or guanine to provide the cells with a substrate for guanylate synthesis by direct salvage, bypassing the synthetic pathway from IMP.

In cell-free extracts, IMPD is known to be subject to negative feedback inhibition by XMP and GMP and to require NAD as a cofactor.^{20,21} These characteristics of the enzyme have been used as the basis for designing nucleoside analogues that function as selective IMPD inhibitors, such as tiazofurin.²² In intact cells, however, this enzyme may be subject to a variety of additional controls at the level of enzyme synthesis, degradation, or posttranslational modification, and it is possible that the changes observed in IMPD

activity in cells treated with retinoic acid or dimethylformamide reflect such control mechanisms. In any case, our studies suggest that such controls of IMPD activity deserve further investigation, since they may be relevant both to normal and to disordered regulation of myeloid cell maturation. Our studies also support the suggestion of others^{11,23} that IMPD is an appropriate target for the design of new chemotherapies that may have selective antineoplastic effects.

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Recombinant Tumor Necrosis Factor Causes Activation of Human Granulocytes

By James W. Larrick, Delores Graham, Karen Toy, Leo S. Lin, George Senyk, and Brian M. Fendly

We have tested the hypothesis that tumor necrosis factor (TNF), by binding to and activating granulocytes, may contribute to the pathogenesis of gram-negative sepsis and the adult respiratory distress syndrome (ARDS). Buffy coat granulocytes incubated with as little as 0.5 ng/mL of recombinant TNF (rTNF) showed a dose-related increase in nitroblue tetrazolium dye reduction, in granulocyte polarization, in superoxide anion release, and in visually apparent aggregation. Purified lipopolysaccharide (1 μ g/mL) caused polymorphonuclear (PMN) aggregation and activation that

was neutralized by polymyxin B. The release of superoxide was augmented by preincubation of the PMNs with γ -interferon. The effect of TNF was neutralized by TNF-specific murine monoclonal antibodies but not by polymyxin B. Scatchard analysis of ^{125} I-rTNF binding to granulocytes revealed about 1,200 receptors per cell with a K_d of 4.9×10^{-10} mol/L. These results suggest that the release of TNF by mononuclear phagocytes contributes to granulocyte activation and aggregation during inflammation.

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INFLAMMATION is characterized by the rapid accumulation of granulocytes and monocytes at sites of tissue injury. These cells participate in the destruction and rapid removal of foreign antigens and damaged tissue. A number of agents have been found to cause activation and chemotaxis of phagocytes including complement fragments,^{1,2} products involved in the kinin and coagulation pathways,^{3,4} collagen,⁵ tissue breakdown products, and arachidonic acid metabolites,⁶⁻⁹ in particular, leukotriene B₄,¹⁰ as well as synthetic peptides related to bacterial-derived products.^{11,12} Most of these substances are generated during an inflammatory response.

During the course of work in our laboratory on the pathogenesis of endotoxemia, we found that tumor necrosis factor (TNF) was released by lipopolysaccharide (LPS) stimulation of macrophages. First described by Carswell et al¹³ in the serum of mice injected with bacillus Calmette-Guerin vaccine and endotoxin, TNF was noted principally for its cytolytic effects on tumor cells. Work by Beutler and coworkers¹⁴⁻¹⁶ has since suggested that TNF might be an important mediator in the response to injury by mobilizing the host energy reserves. Although C5a is known to be a major cause of activation and aggregation of granulocytes, we postulate that TNF may also contribute to the granulocyte response. In the present studies we show that granulocytes have specific high-affinity receptors for TNF and that low concentrations of TNF cause aggregation and activation of granulocytes. These effects can be blocked by monoclonal antibodies neutralizing TNF.

MATERIALS AND METHODS

Reagents. *Escherichia coli* 0111:B4 LPS was purchased from LIST Biologicals (Campbell, CA). Nitroblue tetrazolium salt (NBT), polymyxin B, formylmethionylleucylphenylalanine

(FMLP), cytochalasin B, superoxide dismutase (SOD), dexamethasone, and ferricytochrome c (horse heart VI) were purchased from Sigma Chemical Co (St Louis).

Cold C5a was kindly provided by Dr Steven Kunkel, University of Michigan, Ann Arbor, MI. Recombinant human TNF (rTNF),¹ interleukin 2, and β -interferon were provided by the Process Group of Cetus Corp, Emeryville, CA. Purified human γ -interferon was obtained from Meloy Laboratories, Springfield, VA.

Antibodies. Rabbit and monoclonal anti-TNF antibodies were generated as previously described.¹⁸

L929 cytotoxicity assay. L929 cells were the kind gift of Dr Lloyd Old, Memorial Sloan-Kettering Cancer Center. Details of the assay for TNF and neutralization by mouse serum and mouse monoclonals are as described.¹⁸

Neutrophil polarization. Polarization of human polymorphonuclear (PMN) leukocytes was performed as described^{19,20} with minor modifications. PMNs were isolated from fresh heparinized blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation. The PMNs were adjusted to 1×10^6 cells/mL of Hanks' buffered saline solution (HBSS), and 0.9 mL was added to each of duplicate tubes (12 $\times$ 75 mm polypropylene) containing 0.1 mL of stimulant or HBSS. The samples were incubated 15 minutes in a 37 °C shaking waterbath. To end the reaction, 1 mL of ice-cold, 0.1 mol/L phosphate-buffered formaldehyde (10% vol/vol), pH 7.2, was added to each tube. Cells were kept cold (4 °C) until they were examined by phase microscopy. Tubes were coded and scored blindly for gross morphological changes indicating polarization. PMNs, round when unstimulated, were considered to exhibit polarization when their shape appeared elongated, often with distinct uropods. The percentage of cells polarized was determined by counting 200 cells per tube.

Ferricytochrome c reduction assay. Superoxide anion production was measured by the ferricytochrome c reduction method.²¹ PMNs (1×10^6 cells) were suspended in HBSS (without phenol red), pH 7.2, in 12 $\times$ 75-mm tubes containing stimulant and ferricytochrome c (80 μ mol/L final concentration) for a final volume of 1 mL. In some experiments PMNs were pretreated with 5 μ g/mL cytochalasin B for five minutes at 37 °C prior to stimulation. Controls containing SOD also were run in parallel. Tubes were incubated at 37 °C for 15 minutes. The reaction was stopped by centrifuging the suspension for ten minutes at 1,200 rpm at 4 °C. Absorbance of the supernatants was then determined using a spectrophotometer at 550 nm. The 550-nm optical density values were converted to nanomoles of reduced cytochrome c using the extinction coefficient 21.0×10^3 (mol/L)⁻¹ cm⁻¹ and expressed as nmoles/10⁶ PMNs/15 minutes.

NBT reduction assay. The buffy coat blood fraction (from a blood bank in a volume of 20 to 30 mL from 1 unit of blood) served as the source of granulocytes. Assays were performed in small, plastic cryogenic vials (Corning Glass Works, Corning, NY) in a volume of 0.5 mL. To 0.25 mL of buffy coat blood was added 0.25 mL of NBT

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dye (Sigma Chemical Co) at 2 mg/mL in saline. Additional ingredients were added in small volumes to achieve the desired concentration, and vials were incubated for 30 minutes at 37 °C. When assaying the efficacy of antibodies, antigen and antibody were preincubated in small volumes (5 to 25 μ L) in 96-well round-bottom Costar plates under conditions of high humidity for 30 minutes at 37 °C before being transferred to assay vials. Following incubation, thin coverslip blood smears were made, air-dried, and stained with Wright-Giemsa stain (Camco Quick Stain, Cambridge Chemical Products, Ft Lauderdale, FL). Smears were then examined under oil immersion and scored for the percentage of NBT-positive PMNs, ie, those containing black deposits of reduced NBT dye in their cytoplasm. The baseline NBT reduction, ie, with no stimulus, was less than 1% in all experiments. The biochemical basis of this assay has been defined.²²

TNF binding assay. TNF was labeled to a specific activity of 20 μ Ci/ μ g of protein using the iodogen method (Pierce Chemical Co, Rockford, IL). Binding assays were performed on 5×10^6 cells using various concentrations of labeled TNF. Binding assays were carried out for four hours at 4 °C. Cells were separated from unbound ¹²⁵I-TNF by three washes in 2% sucrose in phosphate-buffered saline. In all experiments, a 100-fold excess TNF was added, and the nonspecific ¹²⁵I-TNF binding was subtracted.

RESULTS

To rule out the possibility that TNF was directly cytotoxic to granulocytes, we incubated PMNs in HBSS with 10 and 1 μ g/mL of rTNF, with FMLP (10^{-6} mol/L), and with no additives. Cell viability was observed using both trypan blue dye exclusion and ethidium bromide/acridine orange staining. No differences in viability were observed among the four cell populations over a period of 22 hours (data not shown).

Activation of human granulocytes by rTNF. Figure 1 shows that rTNF induces a dose-dependent increase in NBT dye reduction by human granulocytes. A concentration of rTNF as low as 100 pmol/L (1.7 ng/mL = 100 pmol/L) produces granulocyte activation well above the background. No-stimulus controls were less than 1% in all NBT studies (data not shown). Polymyxin B, which blocks LPS-induced

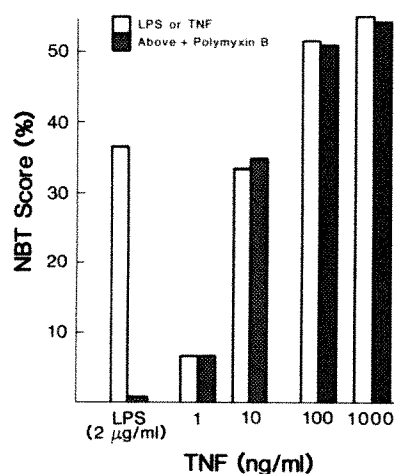


Fig 1. Dose response of rTNF-induced granulocyte NBT dye reduction. Note that the LPS-induced NBT reduction is blocked by polymyxin B (5 mg/mL); the effect of rTNF is not. The assay was read after 30 minutes' incubation. The no-stimulus control values were <1% positive.

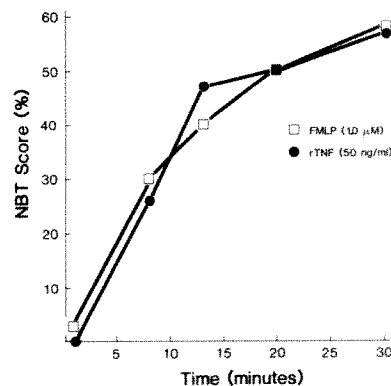


Fig 2. Kinetics of the activation process. The kinetics of FMLP- and rTNF-induced NBT dye reduction are identical. Full induction of NBT dye reduction begins to plateau at 15 minutes. Concentrations of 50 ng/mL rTNF and 1.0 μ mol/L FMLP were used for these studies. The no-stimulus control values were <1%.

NBT dye reduction,^{23,24} did not block the rTNF-induced activation of granulocytes. Interestingly, when concentrations of LPS (2 μ g/mL) and rTNF (100 ng/mL), both of which induced maximal granulocyte NBT dye reduction alone, were added together to the cells, no additional increase in the number of positive cells was observed (data not shown). Aggregation of PMNs was visually apparent at doses of rTNF above 100 ng/mL. No stimulation of NBT reduction with rTNF or LPS was observed in the presence of 1 mmol/L *N*-ethylmaleimide, which suggests that a superoxide-generating oxidase was reducing the NBT.

Figure 2 shows the kinetics of the activation process comparing FMLP (1.0 μ mol/L) and TNF (50 ng/mL). Both substances induce rapid activation, with a plateau beginning after 15 minutes.

rTNF also produces a dose-dependent increase in human granulocyte polarization similar to the response seen with C5a, a known chemotactic factor (Table 1). PMN polarization induced by rTNF was more pronounced than that produced by LPS. Furthermore, the activation induced by rTNF was not reduced by the addition of polymyxin B (Table 2).

To determine whether rTNF contributed to the respiratory burst observed during the activation of PMNs, measurements of the superoxide anion were made by the ferricytochrome c reduction method. Table 3 shows that rTNF caused

Table 1. rTNF-Induced Polarization of PMNs

	rTNF	C5a
10 μ g/mL	86.7 $\pm$ 5.3*	ND
1 μ g/mL	80.3 $\pm$ 3.7	ND
100 ng/mL	67.7 $\pm$ 4.8	92.3 $\pm$ 2.9
10 ng/mL	63.7 $\pm$ 17.1	93.1 $\pm$ 4.1
1 ng/mL	36.8 $\pm$ 18.7	45.0 $\pm$ 6.2
100 pg/mL	7.3 $\pm$ 1.1	9.7 $\pm$ 5.6
HBSS	2.9 $\pm$ 1.5	
FMLP (10^{-7} mol/L)	94 $\pm$ 2.6	

Abbreviation: ND, not determined.

*PMN polarization (percentage $\pm$ SD), mean $\pm$ SE of four experiments.

Table 2. Effect of Polymyxin B on Polarization of PMNs Induced by LPS or rTNF

	Addition of Polymyxin B (5 µg/mL)	
	—	+
LPS		
1 µg/mL	46.0*	15.8
100 ng/mL	165.5	7.5
10 ng/mL	4.0	6.5
rTNF		
100 ng/mL	65.8	66.0
10 ng/mL	59.0	63.5
1 ng/mL	53.0	54.0

*PMN polarization (%), mean of three experiments. The addition of media alone and polymyxin B alone had ≤6.0% positive cells.

a two- to threefold increase in the release of superoxide anion. Furthermore, the stimulation was augmented by preincubation of the PMNs with 100 units of γ -interferon. Note that these studies were performed in the absence of cytochalasin B, which is frequently used to augment O_2^- release. When cytochalasin B is added to the cells prior to stimulation, there is a modest increase in TNF stimulation at the higher concentrations (Table 4). Cells stimulated by FMLP in the presence of cytochalasin B released up to 20-fold more O_2^- .

Neutralization of rTNF activation of granulocytes by antibodies. Both rabbit (Fig 3) and mouse (Fig 4) antisera, which neutralized the in vitro cytotoxicity of rTNF, blocked rTNF-induced NBT dye reduction by granulocytes. In addition, a series of murine anti-TNF monoclonal antibodies was tested. These monoclonals were shown to neutralize rTNF-induced mouse L cell cytotoxicity¹⁸ as well as rTNF-induced granulocyte NBT dye reduction (Fig 4).

Binding of rTNF to human granulocytes. Human granulocytes demonstrated rapid saturable binding of ¹²⁵I-labeled rTNF. Scatchard analysis revealed a low number (1,174 sites/cell) of high-affinity ($K_d = 5 \times 10^{-10}$ mol/L) receptors on these cells (Fig 5). This binding was inhibited by a 100-fold molar excess of cold rTNF but not by a 100-fold molar excess of human C5a, recombinant interleukin 2, or β -interferon (data not shown).

DISCUSSION

These studies demonstrate that purified human rTNF binds specifically to high-affinity receptors on PMN leuko-

Table 4. rTNF-Induced Generation of Superoxide Anion in the Presence of Cytochalasin B

	Control	Cytochalasin B (5 µg/mL)*
FMLP, 10^{-7} mol/L	1.1 ± 0.5 †	26.7 ± 3.1
TNF, 1 µg/mL	2.8 ± 0.7	6.3 ± 0.7
100 ng/mL	3.3 ± 0.8	4.3 ± 0.6
10 ng/mL	2.3 ± 0.4	2.4 ± 0.4

The values are the mean and SE of six experiments without cytochalasin B and three experiments with cytochalasin B.

*Cells were preincubated for ten minutes 37 °C with cytochalasin B prior to the addition of stimulants.

†Values are expressed in $nm\ O_2^-/10^6$ PMNs/15 min minus the cell control values ($\pm$ SE).

cytes and that this binding leads to a dose-dependent increase in the activation of these granulocytes. The concentration of rTNF required to elicit PMN cell polarization and the reduction of NBT dye is as low as 100 pmol/L, which compares favorably with the receptor affinity measured on these cells and on other cells tested in our labs and by other investigators.²⁵⁻²⁷ The concentrations of rTNF required to induce visually apparent aggregation and production of significant amounts of superoxide anion are up to tenfold higher. A number of laboratories have investigated this dissociation of membrane polarization and the oxidative burst.²⁸⁻³⁰ For example, Sklar et al³¹ showed that the time of receptor occupation by FMLP determined the relative membrane potential changes and the amount of superoxide generated. Longer receptor occupancy or a higher percentage of receptor occupation was required for the generation of O_2^- . γ -Interferon modestly sensitizes neutrophils to the effects of low concentrations of TNF. Synergism has been shown by workers studying TNF-induced granulocyte-mediated cytotoxicity³² and TNF-mediated tumor killing.³³ Studies by two groups^{34,35} have recently shown that γ -interferon modestly enhances the expression of TNF receptors. The low concentrations of TNF required to stimulate the respiratory burst are within the range commonly found in cultures of stimulated macrophages (J.W. Larrick, unpublished data) and are probably physiologically relevant at the sites of inflammation. Although researchers have commonly used cytochalasin B to increase the detection of released O_2^- by FMLP-stimulated PMNs, our studies show that this agent had only

Table 3. rTNF-Induced Superoxide Production by PMNs

Stimulant	10^6 PMNs (nmol/15 min)	10^6 PMNs + γ -IFN (nmols O_2^- /15 min)
Media	$1.9 \pm .11$ *	$1.19 \pm .70$
FMLP, 10^{-7} mol/L	$3.0 \pm .53$	$6.51 \pm .49$
TNF, 1 µg/mL	$4.7 \pm .72$	5.81 ± 1.03
TNF, 100 ng/mL	$5.2 \pm .85$	6.41 ± 1.20
TNF, 10 ng/mL	$4.2 \pm .47$	$4.48 \pm .74$
TNF, 1 ng/mL	$2.7 \pm .26$	$3.21 \pm .31$

PMNs at a 10^6 /mL concentration were preincubated at 37 °C for 20 minutes with either HBSS or 100 U/mL γ -interferon, then tested for their response to rTNF. The data are presented as nmol/ 10^6 PMN/15 min. There was no cytochalasin B preincubation in these experiments.

*Mean $\pm$ SE of six experiments.

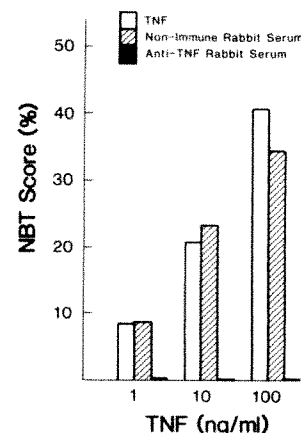


Fig 3. Neutralization of rTNF-induced NBT dye reduction by rabbit anti-TNF antiserum. Following preincubation (30 minutes) with rTNF (final assay concentrations are shown in the Fig) in small equal volumes, the α TNF serum was at a 1:20 dilution during the 30-minute assay. The no-stimulus control values were <1% positive.

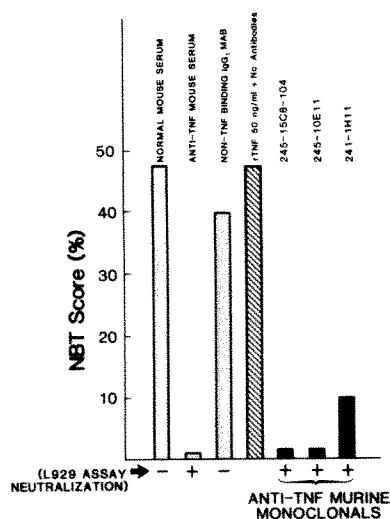


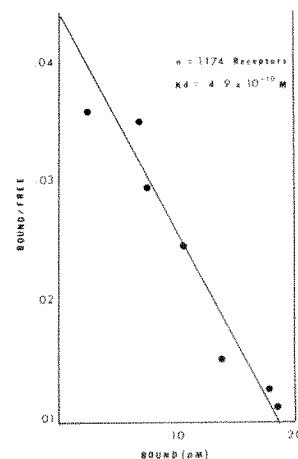
Fig 4. Neutralization of rTNF-induced NBT dye reduction by mouse anti-TNF antiserum and anti-TNF monoclonal antibodies. Following preincubation (30 minutes) with rTNF (final assay concentrations of 50 ng/mL) in small equal volumes, monoclonals were in a 4 mol/L excess to rTNF and mouse anti-TNF serum at 1:20 dilution during the 30-minute assay. Normal mouse serum and the control IgG, monoclonal do not neutralize the cytotoxic effect of rTNF in the L929 assay. Anti-TNF mouse serum and the three monoclonals all neutralize rTNF in the L929 assay. The no-stimulus control values were <1%.

modest effects on the O_2^- released by TNF-stimulated PMNs.

Our studies show that TNF binds to a low number of high-affinity sites on PMN leukocytes. This binding is both specific and saturable. Similar dissociation constants and numbers of TNF receptors have been reported by others²⁵ using various cell lines. Because TNF is known to bind to both sensitive and insensitive cell lines, it will be important to better understand how receptor occupancy triggers normal cells to respond and certain neoplastic cell lines to die. The 5-lipoxygenase pathway has been implicated in receptor-response coupling of PMNs,³⁶⁻³⁸ but to date our studies with the arachidonate inhibitors ETYA, BW755, indomethacin, and nordihydroguaiaretic acid (NDGA) have given little or no inhibition of TNF-induced activation (data not shown). Studies of other second-messenger pathways are in progress.

Our work suggests that TNF is a member of a growing list of inflammatory mediators that bind to and cause activation of granulocytes. It also provides evidence that mononuclear

Fig 5. Scatchard analysis of ^{125}I -rTNF binding to human granulocytes. Granulocytes (5×10^6) were incubated with labeled rTNF for 14 hours at 4°C. Counts bound in the presence of 100-fold excess cold rTNF were subtracted from counts bound to give the specific rTNF bindings.



phagocytes may stimulate granulocytes via TNF during inflammation. Other groups have recently shown that TNF stimulates binding to endothelial cells³⁹ as well as granulocyte phagocytosis.^{32,40} Clearly, there is a complex interplay of these recently cloned cytokines in the inflammatory response. The recent work of Beutler et al¹⁵ is interesting in this regard. These workers showed that in a murine model of LPS toxicity the lethal effect of endotoxin can be reduced by rabbit antimurine TNF (cachectin) antibodies. If TNF is a major mediator of endotoxemia, its effects on granulocytes and endothelial cells may contribute to the pathogenesis of septic shock and the adult respiratory distress syndrome (ARDS). To date, activation of the alternate pathway of complement with the release of C5a has been thought to be the major stimulant of granulocyte aggregation in this context.⁴¹⁻⁴³ The present studies show that TNF must be included with mediators such as LTB₄, platelet-activating factor, and C5a, which contribute to the aggregation of granulocytes with the subsequent compromise of pulmonary function in gram-negative sepsis and ARDS. Whether the effect of rTNF on PMNs observed in these studies contributes to its antitumor effect or to its toxicity is currently under investigation. From a therapeutic standpoint, the inhibition of the inflammatory effects of TNF may be as important as its direct antitumor effects.

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Studies of a Mechanism Inhibiting the Initiation of the Extrinsic Pathway of Coagulation

By L. Vijaya Mohan Rao and Samuel I. Rapaport

We have extended earlier studies (Blood 66:204, 1985) of a mechanism of inhibition of factor VIIa/tissue factor activity that requires a plasma component (called herein extrinsic pathway inhibitor or EPI) and factor Xa. An activated peptide release assay using ^3H -factor IX as a substrate was used to evaluate inhibition. Increasing the tissue factor concentration from 20% to 40% (vol/vol) overcame the inhibitory mechanism in normal plasma but not in factor VII-deficient plasma supplemented with a low concentration of factor VII. A second wave of factor IX activation obtained by a second addition of tissue factor to plasma with a normal factor VII concentration was almost abolished by supplementing the reaction mixture with additional EPI and factor X. Factor Xa's active site was

necessary for factor Xa's contribution to inhibition, but preliminary incubation of factor Xa with EPI in the absence of factor VIIa/tissue factor complex or of factor VIIa/tissue factor complex in the absence of EPI did not replace the need for the simultaneous presence of factor Xa, factor VIIa/tissue factor, calcium, and EPI in an inhibitory reaction mixture. Inhibition of factor VIIa/tissue factor was reversible; both tissue factor and factor VIIa activity could be recovered from a dissociated, inhibited factor VIIa/tissue factor complex. EPI appeared to bind to a factor VIIa/tissue factor complex formed in the presence of factor Xa but not to a factor VIIa/tissue factor complex formed in the absence of factor Xa.

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KKNOWN INHIBITORY mechanisms regulating blood coagulation include neutralization of the serine protease enzymes—factors IXa, Xa, and thrombin—by the protease inhibitors antithrombin III and heparin cofactor II and inhibition of the cofactors—factor VIIIa and Va—by activated protein C. These inhibitory reactions modulate the intermediate and late steps of blood coagulation. Little is yet known about inhibitory mechanisms that modulate the initiation of blood coagulation following exposure of blood to tissue factor (ie, initiation of coagulation via the extrinsic pathway). However, it has been known since 1947 that a preliminary incubation of tissue factor with serum would abolish its otherwise lethal effect after intravenous injection into mice.^{1,2} Moreover, it was shown in the early 1950s that the coagulant activity formed after the addition of tissue factor to serum in vitro diminished rapidly on further incubation.³

In a recent report from this laboratory, a plasma-inhibitory material was described that inhibits factor VIIa/tissue factor activity only when factor X is also present in reaction mixtures.⁴ We report herein the results of experiments carried out to explore further the mechanism of this inhibition.

MATERIALS AND METHODS

Reagents. Hereditary clotting factor-deficient plasmas were obtained from George King Bio-Medical, Overland Park, KS. Rabbit brain cephalin was obtained from Sigma Chemical Co (St Louis). $\text{Na}^3[\text{H}]$ borohydride from Amersham Corp (Arlington Heights, IL), Sephacryl S-200 and CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Piscataway, NJ), diethyl aminoethyl Affi-gel blue and all electrophoretic reagents from Bio-Rad Laboratories (Richmond, CA). Unless otherwise specified, the buffer used to dilute reagents contained 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.5, and 1 mg/mL bovine serum albumin (TBS/BSA).

Plasmas. Normal pooled plasma and barium-absorbed plasma were prepared as described earlier.⁴ For some experiments, factor VII-deficient plasma and barium-absorbed plasma were passed through an anti-factor VII column, and factor X-deficient plasma was passed through an anti-factor X column to remove traces of factor VII and factor X from these plasmas, respectively.

Tissue factor. Crude tissue factor preparations were saline extracts of human brain. The standard reagent, prepared as

described earlier,⁴ clotted normal plasma in 13 seconds. A particulate tissue factor reagent, with activity that could be pelleted by centrifugation at 12,000 g for ten minutes, was prepared as described by Bajaj et al.⁵ This tissue factor clotted normal plasma in 22 seconds. A purified tissue factor reagent was used for selected confirmatory experiments. It was prepared by reconstituting purified tissue factor apoprotein, isolated by a modified factor VII affinity technique to be described elsewhere, with a phospholipid mixture containing phosphatidyl ethanolamine, phosphatidyl choline, and phosphatidyl serine (1:1:0.3). This reconstituted, purified tissue factor was diluted in buffer to a concentration that clotted normal plasma in 18 seconds.

Purified human clotting factors. Human factors VII,⁶ IX, and X⁷ were purified to homogeneity by methods described earlier from this laboratory. Purified clotting factor concentrations were determined spectroscopically at A (absorbance) 280 nm using an $E_{\text{cm}}^{1\%}$ (extinction coefficient) of 13.9 for factor VII,⁸ 13.2 for factor IX,⁹ and 11.6 for factors X and Xa.⁹

Factor Xa. This was prepared by incubating purified factor X with insolubilized Russell's viper venom in the presence of calcium as described earlier.⁷ It was stored at -20°C in 50% vol/vol glycerol and diluted to the desired concentration in buffer just before use. The factor Xa had a specific activity of approximately 1,300 U/mg as measured in a one-stage factor Xa assay.¹⁰ It contained 5% to 10% residual zymogen factor X as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

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Factor VIIa. Factor VIIa was prepared just before use by incubating factor VII at a concentration of either 50 $\mu\text{g/mL}$ or 2.5 $\mu\text{g/mL}$ with factor Xa in a 50:1 ratio (wt/wt) for 15 minutes at 37 °C in the presence of 0.42 mmol/L cephalin and 5 mmol/L calcium. In some experiments the factor VIIa preparation was incubated with antibodies to factor X to neutralize the factor Xa activity.

Antibodies to factor VII. Antibodies were raised to purified human factor VII in a goat. The antiserum was adsorbed with barium sulfate (100 mg/mL) and heated to 56 °C for 20 minutes. At a 1,600-fold dilution this antiserum neutralized 50% of the activity of factor VII in normal plasma. It gave a single precipitation line on immunodiffusion against purified factor VII but not against prothrombin, factor X, factor IX, protein C, or protein S. An immunoglobulin fraction was prepared by precipitation at a 45% ammonium sulfate saturation.

Antibodies to factor X. An antiserum to purified human factor X was prepared in a goat and its IgG fraction isolated as described earlier.¹¹ The IgG fraction gave a single precipitation line of identity on a double-immunodiffusion plate with purified factor X and normal plasma. A 100-fold dilution of the IgG fraction neutralized 60% of the factor X clotting activity in normal plasma. Adding the antibody to a reaction mixture of factor Xa, phospholipid, calcium ions, and factor VII prevented any measurable activation of the factor VII by factor Xa.

Preparation of anti-factor VII and anti-factor X columns. The immunoglobulin fraction of anti-factor VII and the purified IgG fraction of anti-factor X were coupled to CNBr-activated Sepharose 4B according to the manufacturer's technical bulletin at approximately 6 and 8 mg/mL packed beads, respectively. Deficient plasmas were passed through respective columns, and the plasma was eluted with TBS. Only those fractions of maximum refractive index were pooled to minimize the dilution of plasma (the plasma was diluted by 10% to 20% in this process).

Sources of the extrinsic pathway inhibitor (EPI). Whole plasma, barium-adsorbed plasma, and a partially purified reagent were used as sources of EPI. The partially purified reagent was prepared from a lipoprotein fraction obtained by ultracentrifugation of plasma to which sodium bromide was added to a final density of 1.21.¹² An apoprotein fraction was obtained by adding 8 vol of cold acetone to 1 vol of lipoprotein fraction and incubating overnight at -20 °C. The precipitate was separated by centrifugation, washed with diethyl ether, pelleted again, dried under nitrogen gas, and then resuspended at one tenth the initial volume in Tris-buffered saline containing 6 mol/L urea at pH 8.0. This suspension was loaded on Sephacryl S-200, which had been equilibrated with the same buffer, and was then eluted with the same buffer. Several protein peaks were obtained; a major protein peak made up primarily of apo-lipoprotein A-I contained most of the EPI. It was concentrated using an Amicon PM-30 membrane (Amicon Corp, Danvers, MA), dialyzed against TBS, and stored at -80 °C.

Preparation of ^3H -factor IX. Siayl ^3H -factor IX was prepared by the general technique of Van Lenten and Ashwell.¹³ The specific activity of different preparations varied between 2 to 4 $\times 10^8$ cpm/mg of protein. Labeled preparations retained 80% to 90% of the biologic activity of nonlabeled preparations as measured in a one-stage factor IX clotting assay.⁷

Measurement of factor IX activation. The rate of factor IX activation was monitored by measuring the ^3H -activation peptide release from the ^3H -factor IX by a modification¹⁴ of the technique described by Zur and Nemerson.¹⁵ Full activation of factor IX, as measured by SDS-polyacrylamide gel electrophoresis, yielded approximately 35% to 40% trichloroacetic acid-soluble counts.

Active site-inhibited factor Xa. An active site histidine-directed serine protease inhibitor, dansyl-glutamyl-glycyl-arginyl-chloro-

methyl ketone (DEGR-CK) was used to inhibit irreversibly the factor Xa activity. One hundred microliters of factor Xa (8.3 $\mu\text{mol/L}$) was incubated with 2 μL of DEGR-CK (7 mmol/L) for 15 minutes at 37 °C, and the excess inhibitor was then removed by dialysis against TBS. This treatment completely inhibited the factor Xa activity as measured in a factor Xa clotting assay.

RESULTS

Evidence that EPI acts against the factor VIIa-tissue factor complex. We knew from the earlier study⁴ that EPI inhibits either tissue factor itself or the factor VIIa/tissue factor complex. Therefore, we carried out an experiment in which the activation of factor IX was measured in factor VII-deficient plasma containing concentrations of tissue factor and of factor VIIa that left only a minimal excess of tissue factor and no significant free factor VII. These concentrations were determined from a preliminary experiment in a system containing ^3H -factor IX, calcium, 0.05 $\mu\text{g/mL}$ factor VIIa, no source of EPI, and increasing concentrations of tissue factor. At this factor VII concentration, increasing the tissue factor concentration above 20% did not increase the rate of activation of factor IX.

Therefore, an experimental reaction mixture was made to contain factor VII-deficient plasma (which had been passed through an anti-factor VII column), 20% (vol/vol) tissue factor, 0.05 $\mu\text{g/mL}$ factor VII, 5 $\mu\text{g/mL}$ ^3H -factor IX, and 5 mmol/L calcium. As shown in Fig 1, a plateau of activation was reached after only 4% of the trichloroacetic acid (TCA) soluble counts were released. The reaction mixture was then divided into aliquots to which first EDTA and then one of the following were added: tissue factor alone (20% vol/vol), factor VII alone (0.20 $\mu\text{g/mL}$), or tissue factor (20% vol/vol) plus factor VII (0.20 $\mu\text{g/mL}$). Following this, each reaction mixture was recalcified. As shown in Fig 1, adding tissue factor alone or adding factor VII alone failed to induce a further significant release of TCA-soluble counts. However, adding both reactants together resulted in a second wave of factor IX activation.

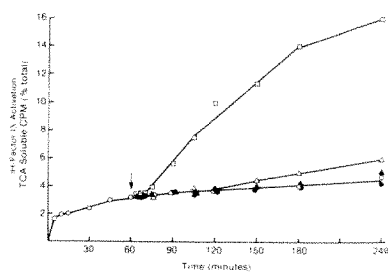


Fig 1. The effect of supplemental tissue factor, factor VIIa, or factor VIIa/tissue factor upon the progress of activation of ^3H -factor IX in factor VII-deficient plasma. The initial incubation mixture (O) contained ^3H -factor IX, 5.0 $\mu\text{g/mL}$; factor VIIa, 0.05 $\mu\text{g/mL}$; tissue factor, 20% (vol/vol); calcium, 10 mmol/L; and factor VII-deficient plasma, 75% (vol/vol). At one hour (arrow) an aliquot was left undisturbed (O), and EDTA, 12 mmol/L, was added to the remaining aliquots. Then the EDTA-treated aliquots were supplemented with one of the following: buffer, (●); factor VIIa, 0.20 $\mu\text{g/mL}$ (Δ); tissue factor, 20% (vol/vol) (Δ); and factor VIIa, 0.20 $\mu\text{g/mL}$, plus tissue factor, 20% (vol/vol) (□). Following this, the reactions were started again in EDTA-treated aliquots with calcium, 22 mmol/L.

In a supplemental experiment, ^{125}I -factor VII/particulate tissue factor/ Ca^{2+} was centrifuged, and the pellet was washed several times in buffer containing calcium to ensure that the persisting radioactivity of the pellet represented only ^{125}I -factor VII complexed with tissue factor. When the pellet was suspended in either buffer or factor VII-deficient plasma containing EDTA and centrifuged again, the majority of the counts were now found not in the pellet but in the supernatant. However, when the pellet was suspended in buffer or factor VII-deficient plasma containing EDTA but excess calcium was added before centrifugation, the majority of radioactivity was found in the pellet. This experiment provides evidence that adding EDTA under our experimental conditions dissociated a factor VIIa/tissue factor/ Ca^{2+} complex and that the dissociated complex could be reassociated by adding calcium again.

Therefore, one may conclude that in the data shown in Fig 1 an inhibited factor VIIa/tissue factor complex was first dissociated and then reassociated. The observation that under these conditions both tissue factor and factor VIIa were required to overcome inhibition by EPI provides strong evidence that EPI inhibits the enzymatic activity of the factor VIIa/tissue factor complex rather than the activity of only its tissue factor component.

Blunting of a second wave of activation of factor IX by supplementing reaction mixtures with additional EPI, factor X, or both. In a reaction mixture made with normal plasma and 20% (vol/vol) tissue factor, a plateau of ^3H -factor IX activation was reached after release of about 5% TCA-soluble counts. Since this reaction mixture contained normal plasma and therefore a tenfold higher factor VII concentration than that used in the reaction mixture of Fig 1, increasing the tissue factor concentration to 40% (vol/vol) allowed free factor VIIa in the mixture to form additional factor VIIa/tissue factor complex. The additional factor VIIa/tissue factor complex initiated a second wave of factor IX activation that was of greater amplitude than the first wave (see the uppermost curve of Fig 2). This suggested that inhibition of the initial factor VIIa/tissue factor complex had consumed inhibitory material in the reaction mixture. When supplemental EPI and factor X were added along with the second aliquot of tissue factor, then the second wave of factor

IX activation induced by additional tissue factor was markedly reduced (see the middle curve of Fig 2).

Adding either EPI or factor Xa alone also diminished the second wave of activation but to a lesser degree. Thus, in three experiments the mean values for TCA-soluble counts at the plateau of a second wave of activation were as follows: when only tissue factor was added, 24%; when tissue factor plus factor X, 8 $\mu\text{g}/\text{mL}$, were added, 18.6%; when tissue factor plus partially purified EPI were added, 17%; and when tissue factor plus both factor X and partially purified EPI were added, 11%.

Failure of active site-inhibited factor Xa to support EPI-induced inhibition. Either factor Xa treated with DEGR-CK to block its active site or untreated factor Xa was added to factor X-deficient plasma that had been passed through an anti-factor X column to remove traces of factor X. ^3H -factor IX, tissue factor, and calcium were added to complete the reaction mixture. The final concentrations were as follows: for factor Xa, 2.5 $\mu\text{g}/\text{mL}$; for tissue factor, 20% (vol/vol). The results were as follows: TCA-soluble counts released after two hours in factor X-deficient plasma with no added factor Xa, 22%; with added DEGR-CK-treated factor Xa, 19%; with added untreated factor Xa, 3.5%.

Failure of preliminary incubation of incomplete reaction mixtures with factor Xa to support the inhibition of the factor VIIa/tissue factor complex. Since the effect of factor Xa appeared to require its active site, we designed an experiment to test the hypothesis that factor Xa's role was enzymatically to convert factor VIIa/tissue factor/ Ca^{2+} into a complex susceptible to EPI. Factor Xa, 2.5 $\mu\text{g}/\text{mL}$, tissue factor, 20% (vol/vol), factor VII, 0.5 $\mu\text{g}/\text{mL}$, and calcium were incubated together for one hour at 37 $^{\circ}\text{C}$, after which anti-factor X antibodies were added to neutralize the factor Xa. After 20 minutes, a source of EPI (factor X-deficient plasma) and ^3H -factor IX were added, and the activation of factor IX was monitored. The activation of factor IX was not inhibited; the extent of activation (release of 30% TCA-soluble counts in two hours) did not differ from that of a negative control in which no factor Xa was added to the reaction mixture. In a positive control in which buffer instead of antibodies to factor Xa was added before the addition of the factor X-deficient plasma, the factor VIIa/tissue factor activity was fully inhibited (release of 4% TCA-soluble counts in two hours).

Additional experiments were carried out to test the hypothesis that factor Xa enzymatically converted EPI from an inert precursor to an active inhibitor. In one experiment (data not shown) factor X-deficient plasma as a source of EPI and factor VII was incubated for 60 minutes with factor Xa and calcium. Antibodies to factor X were then added and, 20 minutes later, tissue factor (20% vol/vol) and ^3H -factor IX. The activation of factor IX was not inhibited.

In a further experiment, barium-absorbed plasma (source of EPI), tissue factor (which also served as a source of phospholipid, a known cofactor for other reactions involving factor Xa), factor Xa, and calcium were incubated for 60 minutes before antibodies to factor X were added. Twenty minutes later, factor VIIa and ^3H -factor IX were added and the activation of factor IX monitored. As can be seen in Fig

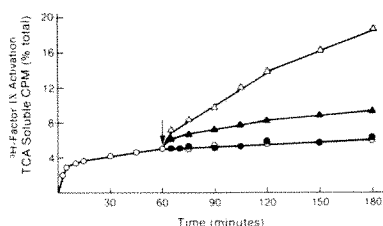


Fig 2. Blunting by partially purified EPI and factor Xa of a second wave of factor IX activation induced in normal plasma by a second addition of tissue factor. The initial reaction mixture (O) contained normal plasma, 75% (vol/vol); tissue factor, 20% (vol/vol); and ^3H -factor IX, 5 $\mu\text{g}/\text{mL}$, and calcium, 10 mmol/L . After 60 minutes (arrow), further reactants were added to the aliquots as follows: buffer (●); tissue factor, 20% (vol/vol) (Δ); or tissue factor, 20% (vol/vol), factor X, 8 $\mu\text{g}/\text{mL}$, and EPI (▲).

3, factor IX was activated at the same rate as in a control mixture made without factor Xa. Thus, no evidence was obtained for a preliminary reaction between EPI and factor Xa, in the presence of phospholipid and calcium as potential cofactors, that bypassed the later need for factor Xa. One should also note in Fig 3 that if all components of the reaction mixture except the substrate were allowed to incubate with factor Xa, then its subsequent neutralization before the addition of the substrate did not impede the inhibition of factor VIIa/tissue factor enzymatic activity.

Binding of EPI to factor VIIa/tissue factor/ Ca^{2+} complex. A factor VII/particulate tissue factor/ Ca^{2+} complex was prepared for use in binding experiments with EPI by adding factor VII to particulate tissue factor in the presence of calcium, separating the pellet by centrifugation, and washing the resuspended pellet several times with calcium-containing buffer. The final resuspended factor VII/tissue factor/ Ca^{2+} complex clotted both recalcified normal plasma and recalcified factor VII deficient plasma in 23 seconds.

This factor VII/tissue factor/ Ca^{2+} complex (25% vol/vol) was incubated with barium-absorbed plasma or buffer (barium-absorbed plasma control), calcium, cold factor IX (5 $\mu\text{g}/\text{mL}$), and either factor Xa or buffer (factor Xa control). (The cold factor IX was included to allow generation of factor VIIa independent of the presence or absence of factor Xa in reaction mixtures.) After incubation for two hours at 37 °C, the factor VIIa/tissue factor complex was isolated by centrifugation and the resultant pellet washed several times in calcium-containing buffer. As shown in Fig 4, when both

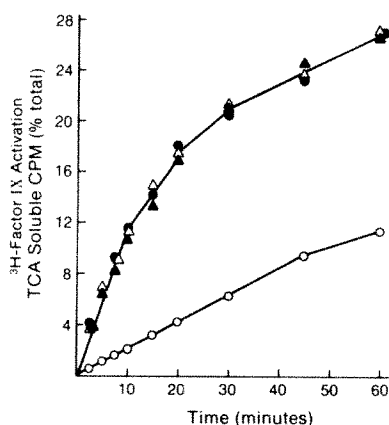


Fig 3. Evidence that tissue factor, factor VIIa, barium-absorbed plasma (source of EPI), Ca^{2+} , must all be present for factor Xa to support inhibition. Barium-absorbed plasma and tissue factor were incubated for one hour at 37 °C with the following additional reactants: (▲) factor Xa, Ca^{2+} ; (●) factor Xa, factor VIIa; (△) factor VIIa, Ca^{2+} ; and, (○) factor Xa, factor VIIa, Ca^{2+} . Then, factor Xa was neutralized with antibodies to factor X (10% vol/vol) for 20 minutes at room temperature. Following this, factor VIIa or Ca^{2+} were added to reaction mixtures that lacked these reactants during the first incubation period. ^3H -factor IX was added to all mixtures at a final concentration of 5.0 $\mu\text{g}/\text{mL}$. The final concentrations of other reactants were as follows: barium-absorbed plasma, 66% (vol/vol); factor VIIa, 0.5 $\mu\text{g}/\text{mL}$; tissue factor, 20% (vol/vol); factor Xa, 2.5 $\mu\text{g}/\text{mL}$; and Ca^{2+} , 10 mmol/L.

barium-absorbed plasma (source of EPI) and factor Xa had been present in the incubation mixture, the resuspended pellet had a markedly diminished ability to activate ^3H -factor IX. In contrast, when control buffer had replaced either barium-absorbed plasma or factor Xa in the incubation mixture, the resuspended factor VIIa/tissue factor complex retained full enzymatic activity. These differences were also demonstrable in a clotting assay; a resuspended, inhibited pellet clotted recalcified factor VII-deficient plasma in 50 seconds whereas resuspended uninhibited pellets clotted the plasma in 26 seconds.

These experiments provided strong indirect evidence that inhibitory activity bound to the factor VIIa/tissue factor complex in the presence of EPI, factor Xa, and calcium. They were incomplete, however, in that they did not test whether EPI could bind to a pellet separated from an incubation mixture lacking factor VII. Therefore, in a further experiment, 0.4 mL of particulate tissue factor preparation was incubated with partially purified EPI or buffer (control) and the following additional reactants: (1) factor VII (0.2 $\mu\text{g}/\text{mL}$), factor Xa (2.5 $\mu\text{g}/\text{mL}$), and calcium; and (2) buffer, factor Xa (2.5 $\mu\text{g}/\text{mL}$), and calcium. After a two-hour incubation, the pellets were isolated, washed, and tested for enzymatic activity in a mixture containing factor VII (0.2 $\mu\text{g}/\text{mL}$), factor Xa (2.5 $\mu\text{g}/\text{mL}$), and ^3H -factor IX. As apparent from the resultant curves of factor IX activation (Fig 5), inhibition of the pellet required not only the presence of factor Xa but also the formation of the factor VIIa/tissue factor/ Ca^{2+} complex. These experiments also suggest the possible binding of inhibitor to the factor VIIa/tissue factor/ Ca^{2+} complex in the presence of factor Xa.

If inhibitory material binds to a pellet, one should be able to demonstrate the loss of inhibitory activity in the supernatant. As the experiments summarized in Fig 6 show, when incubation mixtures made with particulate tissue factor, factor VII, factor Xa, partially purified EPI, and calcium were centrifuged, the resultant supernatant was unable to serve as a source of EPI for a second incubation mixture. In contrast, control supernatants from incubation mixtures lacking factor VIIa or factor Xa retained their ability to

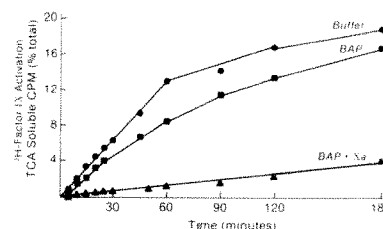


Fig 4. Activation of ^3H -factor IX by resuspended factor VIIa/tissue factor pellets from different types of initial reaction mixtures. Different types of pellets resuspended in calcium-containing buffer were incubated with ^3H -factor IX, 5 $\mu\text{g}/\text{mL}$, and Ca^{2+} , 10 mmol/L, and the activation of factor IX monitored. The pellets were obtained by centrifugation of incubation mixtures containing particulate tissue factor, factor VII, factor IX, Ca^{2+} , and added reactants as follow: buffer (○), barium-absorbed plasma (●), and barium-absorbed plasma and factor Xa (▲). See the text for further details.

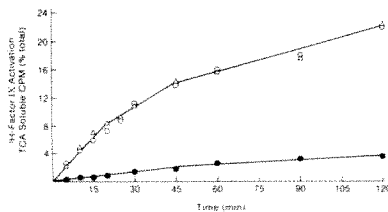


Fig 5. Evidence that a pellet prepared from an incubation mixture containing tissue factor, EPI, factor Xa, and Ca^{2+} , but not containing factor VII, possessed tissue factor activity and did not possess EPI activity. Reaction mixtures contained particulate tissue factor and other reactants as follows: (●) EPI, factor VII, factor Xa, Ca^{2+} ; (○) EPI, factor Xa, Ca^{2+} ; and (Δ) buffer, factor VII, factor Xa, Ca^{2+} . After two hours' incubation at 37 °C, pellets were obtained by centrifugation of incubation mixtures, washed and resuspended in calcium-containing buffer, and tested in the following system: resuspended pellet; factor VII (0.05 $\mu\text{g}/\text{mL}$), factor Xa (2.5 $\mu\text{g}/\text{mL}$), ^3H -factor IX (5.0 $\mu\text{g}/\text{mL}$), and calcium (10 mmol/L).

serve as a source of EPI for a second incubation mixture. These data not only confirm the binding of EPI to the factor VIIa/tissue factor complex but provide further evidence that EPI does not bind to tissue factor alone.

Recovery of tissue factor activity from the inhibited factor VIIa/tissue factor complex. Factor VIIa/tissue factor pellets prepared under conditions that would either inhibit enzymatic activity or would not (Fig 4) and were resuspended in buffer containing 5 mmol/l EDTA, and the particulate tissue factor was pelleted by centrifugation. After being washed several times with EDTA-containing buffer, the pellets were resuspended in calcium-containing buffer. Then, purified factor VII, 0.25 $\mu\text{g}/\text{mL}$, and ^3H -factor IX, 5.0 $\mu\text{g}/\text{mL}$, were added and the activation of factor IX monitored. As can be seen in Fig 7, the tissue factor recovered from an inhibited complex could support the activation of factor IX about as effectively as could tissue factor recovered from uninhibited complexes.

Experiments with purified tissue factor. After a technique was developed to purify tissue factor, selected additional experiments were carried out to confirm results obtained with crude tissue factor preparations. As with the

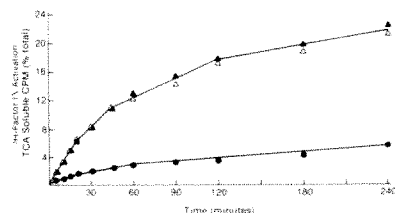


Fig 6. Absence of EPI activity in a supernatant of a reaction mixture made with particulate tissue factor, partially purified EPI, factor VII, factor Xa, and Ca^{2+} . Reaction mixtures contained particulate tissue factor and other reactants as follows: (Δ) EPI, factor VII, factor Xa, Ca^{2+} ; (●) EPI, Ca^{2+} ; and (Δ) buffer, factor VII, factor Xa, Ca^{2+} . After a one-hour incubation at 37 °C, the reaction mixtures were centrifuged, and the supernatants were tested for their EPI activity. The test system was as follows: supernatant; tissue factor, 10% (vol/vol); factor VIIa, 0.05 $\mu\text{g}/\text{mL}$; factor Xa, 2.5 $\mu\text{g}/\text{mL}$; ^3H -factor IX, 5 $\mu\text{g}/\text{mL}$; and Ca^{2+} , 10 mmol/L.

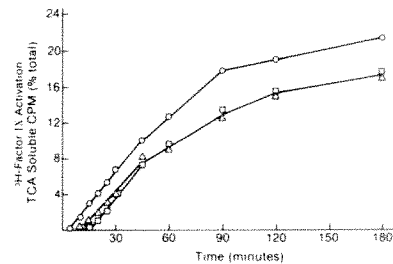


Fig 7. Evidence that tissue factor retains its activity after dissociation from an inhibited factor VIIa/tissue factor complex. After two hours' incubation at 37 °C, the factor VIIa/tissue factor/ Ca^{2+} complex was pelleted from incubation mixtures made with factor VII, particulate tissue factor, factor IX (to activate factor VII in mixtures that did not contain factor Xa), calcium and the following: (○) factor Xa, buffer; (Δ) barium-absorbed plasma, buffer; and (□) factor Xa, barium-absorbed plasma-containing buffer. Then, pellets were resuspended in EDTA-containing buffer to dissociate the tissue factor. The dissociated tissue factor was washed twice in EDTA-containing buffer and resuspended in calcium-containing buffer. The tissue factor was then tested for activity as measured by its ability to support the activation of ^3H -factor IX in a reaction mixture containing factor VII, calcium, and ^3H -factor IX.

crude preparations, both EPI and factor Xa were required to inhibit the enzymatic activity of a factor VIIa/purified tissue factor complex. Furthermore, as with the crude preparation, DEGR-CK-treated factor Xa failed to support EPI-induced inhibition.

DISCUSSION

In earlier work from this laboratory, a plasma mechanism for inhibiting the enzymatic activity of factor VIIa/tissue factor was described that involves both a plasma-inhibitory material and factor Xa.⁴ The earlier data did not allow us to conclude whether inhibition stemmed from neutralization of only the tissue factor component of the complex or from neutralization of the complex as a whole.

The data presented herein now establish that inhibition induced by the plasma-inhibitory material (provisionally called EPI) and factor Xa results from the inhibition of the complex as a whole. Thus, when an inhibited factor VIIa/tissue factor complex was dissociated with EDTA and then reassociated with calcium under conditions in which the reaction mixture contained an excess of free factor VIIa or of free tissue factor but not of both, a new factor VIIa/tissue factor complex with enzymatic activity was not demonstrable. Only when the reaction mixture contained both additional factor VII and tissue factor did recalcified reaction mixtures exhibit new factor VIIa/tissue factor enzymatic activity (Fig 1).

If the reaction mixtures initially contained excess free factor VII, then adding more tissue factor alone induced a second wave of factor VIIa/tissue factor enzymatic activity. Such a second wave often activated more factor IX than did the first wave of activity (Fig 2). However, one could curtail the second wave of enzymatic activity by supplementing the reaction mixture with additional EPI and factor X (Fig 2). It would appear, therefore, that normal plasma contains a

concentration of EPI sufficient to limit extrinsic coagulation initiated by a low concentration of the factor VII/tissue factor complex but insufficient to limit extrinsic coagulation initiated by a higher concentration of the factor VII/tissue factor complex (such as might occur pathologically when the contact of blood with brain tissue after head trauma leads to extensive defibrination¹⁶).

Inhibition of the factor VIIa/tissue factor enzymatic activity does not inhibit tissue factor irreversibly. Tissue factor resuspended from a pellet recovered after dissociating an inhibited factor VIIa/tissue factor complex retained its ability to function as a cofactor in a new factor VIIa/tissue factor complex (Fig 7). Moreover, the supernatant obtained after centrifuging a dissociated, inhibited factor VIIa/tissue factor complex had factor VII(a) coagulant activity. For example, in one experiment the supernatant gave a clotting time in a one-stage factor VII assay of 26 seconds as compared with the inhibited reaction mixture before decalcification, which gave a clotting time of 53 seconds in the assay. However, present data are inadequate to estimate the extent of recovery of factor VII activity in supernatants of decalcified, inhibited complexes. This will require the use of the coupled amidolytic factor VII assay technique¹⁷ to eliminate the differences in measured factor VII activity stemming from a variation in the ratios of factor VIIa to factor VII in supernatants of experimental and control reaction mixtures.

In experiments carried out before the discovery of factor X, Hjort¹⁸ found that the increased coagulant activity resulting from the incubation of "proconvertin reagent," tissue factor, and calcium (which was referred to as convertin) could be inhibited by a material in barium-absorbed serum (which was referred to as anticonvertin). It is now clear that Hjort's "proconvertin reagent" contained both factor VII and factor X and, therefore, that factor Xa was generated in his reaction mixtures. Thus, what we have called EPI almost surely is Hjort's original anticonvertin activity. This possibility receives further support from Hjort's observation¹⁸ that both tissue factor and proconvertin (factor VII) activity could be recovered after decalcification of inhibited "convertin."

Recent observations of inhibition of the factor VIIa/tissue factor enzymatic activity by other investigators also appear related to the EPI-factor Xa mechanism. In 1981, Carson¹⁹ reported that plasma high-density lipoproteins could inhibit the factor VIIa/tissue factor activation of factor X. Since we can recover about 50% of the total EPI activity of plasma in a lipoprotein fraction of plasma, the activity Carson described very probably is EPI. His use of factor X as the substrate for factor VIIa/tissue factor masked the need for factor Xa to observe the inhibitory effect. The experiments on the inhibition of activated factor VII by normal plasma reported by Dahl and coworkers²⁰ are also best explained by an EPI-factor Xa-induced inhibition of the factor VIIa/tissue factor complex. Morrison and Jesty's²¹ report that factor Xa inhibits the tissue factor-dependent activation of factor IX in human plasma can be accounted for by the requirement for factor Xa for the inhibitory effect of EPI. More recently, Hubbard and Jennings²² have also provided data showing the

participation of factor Xa in the inhibition of tissue factor activity by a plasma component (presumably EPI).

The mechanism of factor Xa's participation in the inhibitory reaction is not yet known. Since inhibiting factor Xa's active site abolishes EPI's ability to participate in the inhibitory reaction, it would be logical to assume that factor Xa acts enzymatically. Yet, in experiments in which factor Xa was first incubated with components of incomplete reaction mixtures and then neutralized with anti-factor X antibodies, we could not obtain evidence that factor Xa's role was to act enzymatically upon EPI in the absence of factor VIIa/tissue factor or, conversely, upon factor VIIa/tissue factor in the absence of EPI.

If all components except the factor IX substrate were allowed to incubate together and the factor Xa was neutralized with antibodies before adding the substrate, then factor VIIa/tissue factor enzymatic activity was inhibited (Fig 3). Thus, factor Xa appears to exert its role in a reaction independent of the substrate. The data as a whole are compatible with a hypothesis that factor Xa acts enzymatically upon EPI but only when factor VIIa, tissue factor, and calcium are also present. This hypothesis will be tested further when a more highly purified EPI preparation becomes available.

How EPI induces inhibition is also not yet understood. As already mentioned and, indeed, as Hjort probably first showed in 1957,¹⁸ EPI does not irreversibly inactivate tissue factor. Nor does EPI appear to inactivate factor VII(a). Thus, it is most unlikely that EPI acts enzymatically. The evidence suggesting that EPI is consumed in the inhibitory reaction further supports this conclusion. EPI appears to bind to the factor VIIa/tissue factor complex, for its activity is not demonstrable in the supernatant after centrifugation of an inhibited factor VIIa/tissue factor complex (Fig 6). However, EPI does remain in the supernatant of a pelleted factor VIIa/tissue factor complex formed in a reaction mixture lacking factor Xa. We take these results to mean that binding of EPI to a factor VIIa/tissue factor complex is required for EPI's inhibitory effect. This, plus an apparently limited concentration of EPI in plasma and the evidence that adding a low concentration of tissue factor to plasma leaves a large excess of free factor VII in the plasma,²³ would explain why adding a high concentration of tissue factor to plasma can bypass the inhibitory effect of EPI upon factor VIIa/tissue factor activity.

The bleeding of patients with hemophilia establishes that the extrinsic pathway, acting alone, cannot generate sufficient factor Xa for normal hemostasis. Inhibition of factor VIIa/tissue factor activity triggered by the factor Xa it generates could account for the need for a factor IXa/factor VIIIa/phospholipid activator for continuing generation of factor Xa during hemostasis. If so, the EPI-factor Xa mechanism for the inhibition of factor VIIa/tissue factor enzymatic activity could represent a key regulatory reaction for normal hemostasis.

ACKNOWLEDGMENT

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Cellular and Subcellular Localization of the Bactericidal/Permeability-Increasing Protein of Neutrophils

By Jerrold Weiss and Inge Olsson

Human and rabbit polymorphonuclear leukocytes contain a bactericidal/permeability-increasing protein (BPI), a potent cytotoxin active specifically against gram-negative bacteria. To identify the cell population(s) producing BPI, we have examined mature and immature human blood cells for BPI by immunofluorescence of intact cells and radioimmunoassay and bioassay of cell extracts. By immunofluorescence and radioimmunoassay of cells from peripheral blood, BPI was detected only in neutrophils; immunofluorescent staining was punctate, indicative of the granule localization of BPI. Nearly all (>90%) BPI was recovered during the subcellular fractionation of neutrophils (N_2 cavitation and discontinuous Percoll gradient) in fractions containing primary granules. Little BPI was released from intact cells during degranulation (cytochalasin B and f-Met-Leu-Phe) or could be extracted from isolated granules with salt or weak acid, which suggests that most granule-associated BPI is membrane bound. Double staining of bone

marrow smears for BPI and lactoferrin revealed BPI only in neutrophil precursors including (pro)myelocytelike cells lacking lactoferrin, a marker of neutrophil secondary granules. Of several human cell lines tested, only the promyelocytelike HL-60 (and to a lesser extent, KG-1) cells contained BPI. BPI was present in a more mature subpopulation (<25%) of untreated HL-60 cells, recognized by surface marker analysis (rosetting with IgG-sensitized sheep RBC, the absence of proliferation-associated cell surface antigen). Induction of neutrophilic or monocytic differentiation caused, respectively, a small (approximately 50%) rise or fall in the BPI content. These findings indicate that BPI is a specific product of the neutrophil lineage and, hence, of the specialized cytotoxic apparatus of the neutrophil that plays an essential role in host defense *v* gram-negative bacteria.

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NEUTROPHILIC (heterophilic) polymorphonuclear leukocytes (PMN) are highly specialized phagocytic cells that play a primary role in defense of the host against invading microorganisms.¹ For this purpose, PMN elaborate a variety of microbicidal agents.¹⁻⁵ One of these cytotoxic substances is a membrane-active cationic protein with potent cytotoxic activity directed specifically against several species of gram-negative bacteria.³ We have isolated this protein from human neutrophils and a very similar protein from rabbit PMN.^{6,7} In both species, this bactericidal/permeability-increasing protein (BPI) appears to play an important role in the killing of several species of gram-negative bacteria by PMN.^{3,8,9}

Both human and rabbit BPI are localized in cytoplasmic granules,^{6,10,11} specialized organelles that contribute greatly to the antimicrobial function of PMN.^{1-5,12} Preliminary examination of other phagocytic and nonphagocytic blood cells (bioassay of cell extracts) has suggested that BPI is present predominantly, if not solely, in neutrophilic (heterophilic) PMN.^{6,10} We have now used immunofluorescence and radioimmune assays to demonstrate that BPI is a specific

product of the neutrophil that is synthesized at or around the promyelocyte stage of differentiation and stored in the primary granules. Studies on the promyelocytelike leukemic cell line HL-60 suggest that the synthesis of BPI may occur after that of the primary granule protein myeloperoxidase.

MATERIALS AND METHODS

Cells. Heparinized venous blood from healthy volunteers and bone marrow aspirates collected for diagnostic purposes by the hematology service were obtained after informed consent. Only bone marrow samples with the normal myeloid spectrum were used. Mononuclear and polymorphonuclear leukocytes and erythrocytes were separated by sedimentation on discontinuous density gradients of Ficoll 400/sodium diatrizoate (Sigma Chemical Co, St Louis).¹³ Platelet-rich suspensions were collected from outdated (one to five days) blood obtained from the New York Blood Bank as previously described.¹⁴

All cell lines were maintained in culture at 2×10^4 to 2×10^6 cells/mL in RPMI 1640 medium supplemented with 10% to 20% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

All cells were washed twice with Dulbecco's phosphate-buffered saline supplemented with 0.2% (wt/vol) bovine serum albumin (PBSA) before use. Cell smears were prepared by cytospin (5×10^4 cells in 0.1 mL PBSA; Shandon Southern Instruments, Inc, Sewickley, PA) and stained (May-Grünwald-Giemsa) for differential counting. Eosinophils and basophils were also identified by their characteristic granular appearance seen by phase-contrast microscopy.

Differentiated HL-60 cells were recognized by the reduction of nitroblue tetrazolium after stimulation with phorbol myristate acetate (1 μ g/mL)¹⁵ and by the expression of nonspecific esterase activity.¹⁶

Fractionation of HL-60 cells by rosetting with IgG-coated sheep erythrocytes. HL-60 cells were incubated with an equal number of sheep erythrocytes that had been pretreated with a subagglutinating dose of rabbit (antisheep erythrocyte) antiserum. After the incubation (ten minutes at 4 °C followed by centrifugation at 50 to 100 g for five minutes and an additional 60-minute incubation at 4 °C), rosetting and nonrosetting cells were separated by sedimentation in a density gradient of Isopaque-Ficoll. After separation, erythrocytes were lysed by brief treatment with distilled water.

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Antibodies and immunoblotting. Antisera to human BPI and myeloperoxidase were obtained after immunization of New Zealand White rabbits with purified BPI⁶ and myeloperoxidase,¹⁷ respectively. Preimmune sera were collected from the same rabbits before immunization. The mouse monoclonal antibody Y201¹⁸ was generously provided by Dr Mary B. Todd (Department of Medicine, Yale University School of Medicine, New Haven, CT).

The specificity of the rabbit (anti-BPI) serum was confirmed by Western blot analysis of the serum against a crude extract of neutrophils (Fig 1). Neutrophils (10^8 /mL) were extracted with 1% Triton X-100/1% sodium deoxycholate/1 mmol/L phenylmethane-sulfonyl fluoride by sonication for 10 seconds at 40 W (4°C) followed by a 60-minute incubation at 15 to 20°C. Solubilized proteins were collected by centrifugation at 20,000 g for 20 minutes, separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels,¹⁹ and transferred to nitrocellulose by electroblotting.²⁰ The nitrocellulose paper containing transferred proteins was then treated sequentially with 3% BSA in 10 mmol/L Tris-buffered saline (TBS), pH 7.4; anti-BPI serum diluted 100-fold in 3% BSA-TBS containing 0.5% Nonidet P-40 (NP40)/5 mmol/L EDTA; and, after washing with TBS, [¹²⁵I]goat (antirabbit IgG) F(ab')₂ (New England Nuclear, Boston; 100,000 cpm/mL) in 3% BSA-TBS and 0.5% NP40/5 mmol/L EDTA. After washing in TBS, the paper was dried and exposed using Kodak X-Omat AR film.

Immunofluorescence. For detection of intracellular (granule) proteins, cell smears (cytospin) were fixed for one minute at 4°C in buffered formalin acetone.²¹ The fixed smears were washed in PBS, rinsed with PBSA, and overlaid for ten minutes at room temperature with 10% heat-treated pooled human serum to occupy the nonspecific binding sites. After rinsing with PBSA, the smears were overlaid for 30 minutes with rabbit (anti-BPI) or (antimyeloperoxidase) serum diluted 100- to 300-fold in PBSA. After washing, the smears were overlaid for 30 minutes with rhodamine-labeled swine (antirabbit Ig) Ig (Accurate Chemical & Scientific Corp, Westbury, NY) diluted 80-fold with PBSA. Excess rhodamine-labeled Ig was removed in three washes (30 minutes) with PBS. For double staining of BPI and lactoferrin, the smears were then soaked in 1% rabbit

serum (to occupy the remaining binding sites on swine [antirabbit Ig] Ig), rinsed in PBSA, overlaid for 30 minutes with 1% rabbit (antihuman lactoferrin) F(ab')₂ conjugated with fluorescein isothiocyanate (FITC), and washed with PBS.

For double staining of BPI and the cell surface antigen recognized by the mouse monoclonal antibody Y201, unfixed cells were treated in suspension at 4°C with the murine monoclonal¹⁸ and, after washing, with FITC-labeled goat (antimouse IgG plus IgM) Ig (Accurate Chemical & Scientific Corp). After washing, cell smears were prepared by cytopsin and then fixed and stained for BPI as was described.

After staining, the smears were covered with glycerol:PBS (9:1, vol/vol) containing 1 mg/mL *p*-phenylenediamine²² and a coverslip and examined under oil using a Leitz Orthoplan fluorescent microscope (E. Leitz, Inc, Rockleigh, NJ) with excitation and barrier filters for FITC and rhodamine staining. Photographs were taken with Kodak Tri-X pan (ASA 400) film at approximately 1,000× magnification.

Degranulation. Neutrophils (4.5×10^7 /mL) were incubated at 37°C in 20 mmol/L Hepes-buffered saline containing 45% (vol/vol) Hanks' buffered solution (M.A. Bioproducts, Walkersville, MD), pH 7.4, for five minutes in the presence and absence of 5 µg/mL cytochalasin B (Aldrich Chemical Co, Milwaukee) and then for 20 minutes with or without f-Met-Leu-Phe (Sigma Chemical Co). Cells and extracellular medium were separated by centrifugation (250 g, 10 minutes). Cells were extracted either with 0.2% Triton X-100 or as described for immunoblotting.

Subcellular fractionation. Primary and secondary granules of the neutrophils were separated as previously described²³ by N₂ cavitation of purified neutrophils and sedimentation of the postnuclear supernatant in a discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient. The fractions were collected from the top of the gradients with a Densi-Flow pump (Buchler Instruments, Fort Lee, NJ). The particulate fractions were localized by measuring the absorbance at 450 nm, and fractions corresponding to separated α and β bands were pooled.

Extraction of granule proteins. A mixed granule-rich fraction of neutrophils was collected by sedimentation (10,000 g, 20 minutes) of the postnuclear supernatant obtained after N₂ cavitation. The granule-rich pellet was resuspended at 2×10^8 cell equivalents/mL in various media (see Table 2 below) by sonication (10 seconds at 40 to 50 W at 4°C) and then incubated at 4°C for one hour. Solubilized material was isolated by centrifugation at 23,000 g for 20 minutes. The pellet was resuspended as before, and the extraction was repeated. The remaining insoluble residue was extracted with 0.2% Triton X-100 or 1% Triton X-100/1% sodium deoxycholate before assay.

Radioimmunoassay. BPI, myeloperoxidase, and lactoferrin were measured in Triton X-100/sodium deoxycholate extracts of whole cells or subcellular fractions by dot blotting on nitrocellulose following essentially the same procedure as described for immunoblotting and elsewhere.⁹ Rabbit (antihuman lactoferrin) Ig was purchased from Accurate Chemicals & Scientific Corp. Standard curves with purified BPI, myeloperoxidase and lactoferrin (Calbiochem, La Jolla, CA) were used to quantitate the protein content in crude extracts. The amount of [¹²⁵I]-goat (antirabbit Ig) F(ab')₂ bound to strips treated with preimmune serum or in the absence of added antigen was <5% of the maximum binding to strips treated with immune serum and antigen and was subtracted for quantitation.

Bioassay of BPI. The BPI content of whole cell extracts was also determined by measuring the bactericidal activity of 0.16 N H₂SO₄ extracts toward BPI-sensitive *Escherichia coli* S15⁶ in comparison with that of purified BPI. The BPI content assessed by bioassay and radioimmunoassay matched closely. The bactericidal activity toward *E. coli* of crude extracts could be blocked by goat (anti-BPI)

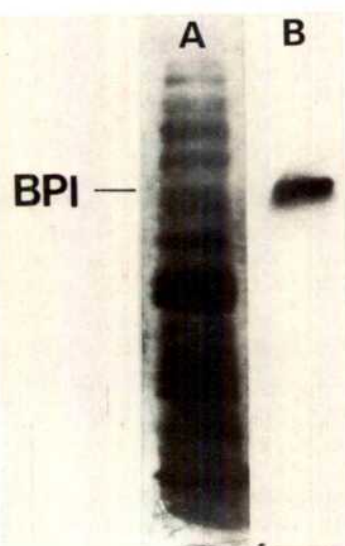


Fig 1. Specificity of rabbit (anti-BPI) serum shown by immunoblotting. A crude extract of neutrophils was electrophoresed in duplicate in a sodium dodecyl sulfate-(10%) polyacrylamide gel. (A) The sample was stained with Coomassie blue. (B) The sample was analyzed by immunoblotting as described in Materials and Methods. Migration of BPI is indicated.

IgG,⁸ thereby confirming that the bioactivity was specifically attributable to BPI.

Miscellaneous. Protein was measured by the method of Lowry et al²⁴, using BSA as a standard. Elastase was measured by hydrolysis of methoxysuccinyl-(L-alanyl)₂-L-prolyl-L-valyl-*p*-nitroanilide.²⁵ Purified BPI was radioiodinated using iodogen-coated beads (Pro Tag; J.T. Baker Co, Philadelphia).²⁶

RESULTS

BPI is present exclusively in neutrophils. To identify blood cells that contain BPI, we treated fixed smears of mixed human peripheral blood cells with rabbit anti-BPI serum and, after washing, rhodamine-labeled swine (anti-rabbit Ig) antibodies. After this treatment, only neutrophils were brightly fluorescent (Fig 2A and B, Table 1). Lymphocytes, monocytes, erythrocytes, platelets, and basophils showed little or no fluorescence. Eosinophils exhibited pale fluorescence (Fig 2A), but as previously noted by others,²⁷ this was nonspecific (ie, of equal intensity after treatment of the cells with immune or preimmune serum). Moreover, unlike the rhodamine staining of neutrophils, staining of eosinophils was also visible with filters for fluorescein excitation and emission. Neutrophil staining was very similar from cell to cell and from individual to individual ($n > 50$) and typically had a punctate appearance consistent with the localization of BPI in cytoplasmic granules.^{6,11} No staining of neutrophils was seen when the cells were treated with either preimmune rabbit serum or immune serum depleted of anti-BPI antibodies by pretreatment with purified BPI.

The BPI content of human neutrophils was assessed more quantitatively by radioimmunoassay of purified neutrophil preparations (Table 1). Neutrophils contain $65 \pm 6.3 \mu\text{g}$ of BPI/ 10^8 cells (range, 41 to $88 \mu\text{g}/10^8$ cells; $n = 5$) representing $0.6\% \pm 0.1\%$ of the total neutrophil protein. In contrast, mononuclear leukocytes and platelet-rich suspensions contain virtually no BPI-like immunoreactive material (Table 1).

Localization of BPI in primary granules of neutrophils. To determine in which of the two main granule populations of the neutrophils BPI is localized, postnuclear supernatants of disrupted neutrophils were subjected to density gradient centrifugation in discontinuous Percoll gradients. As originally described,²³ this procedure yielded two granule-rich fractions (α , [$\rho = 1.13 \text{ g/mL}$] and β [$\rho = 1.08 \text{ g/mL}$]) in which the primary and secondary granules were nearly completely separated as judged by the distribution of myeloperoxidase and lactoferrin (Table 2).²³ BPI was recov-

Table 1. Localization of BPI in the Neutrophils of Peripheral Blood

Cells	Immunofluorescence (% Positive)	RIA ($\mu\text{g BPI}/10^8$ Cells)
Neutrophils	100	65 ± 6
Monocytes	0	$0.6 \pm 0.5^*$
Lymphocytes	0	—
Platelets	0	<0.3
Erythrocytes	0	NT
Eosinophils	0	NT
Basophils	0	NT

Abbreviations: RIA, radioimmunoassay; NT, not tested.

*Eighty percent lymphocytes, 20% monocytes, with 1% contamination of neutrophils.

ered almost exclusively in the α fraction (Table 2), which indicates that it is localized in the primary granules.

In comparison with myeloperoxidase, lactoferrin, and elastase (another primary granule protein), very little BPI is released into the extracellular medium during treatment of intact neutrophils with cytochalasin B and f-Met-Leu-Phe (Table 2). That BPI is more tightly associated with the granules than are these other granule proteins is also indicated by the finding that treatment of isolated granules with either salt (0.15 to 1.0 mol/L NaCl) or weak acid (0.2 mol/L sodium acetate/acetic acid [pH 4.0]) releases nearly all the myeloperoxidase, lactoferrin, and elastase in soluble form but only a small proportion of BPI (Table 2). Purified ¹²⁵I-BPI added to extracted granules is recovered in the soluble phase, thereby indicating that the granule-associated BPI is tightly membrane bound and not artifactually trapped in the insoluble granule residue. Treatment with 1% Triton X-100 and sodium deoxycholate solubilizes BPI as assessed by radioimmunoassay and Western blots (data not shown).

Immunofluorescent staining of BPI in bone marrow cells. Because the primary granules are formed before the secondary granules,²⁸ BPI may be expected to appear before lactoferrin during myeloid differentiation. Indeed, double immunofluorescent staining of BPI and lactoferrin in human bone marrow cells revealed BPI-positive/lactoferrin-negative precursor cells (Figs 3A and B) that morphologically resembled early myeloid precursors (eg, [pro]myelocytes) that contain only primary granules. All lactoferrin-positive cells also stained positively for BPI and resembled later myeloid precursors that contain both primary and secondary granules (Figs 3A and B).

Presence of BPI in promyelocytelike cell line(s). To substantiate further that BPI is a specific product of the

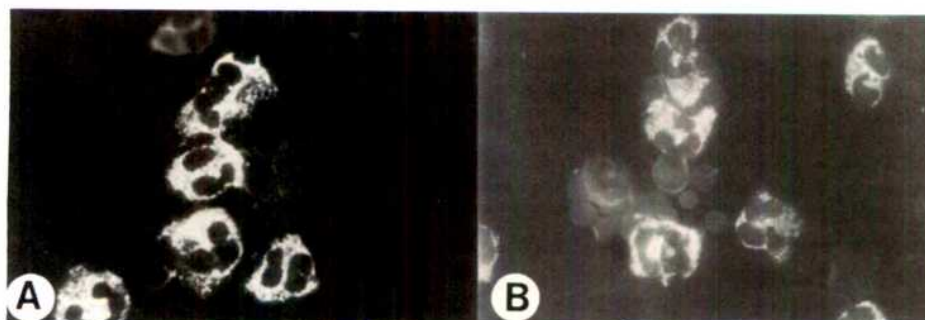


Fig 2. Immunofluorescent staining of BPI in cells from human peripheral blood. All neutrophils and only neutrophils stain brightly. (A) The cell at the top is an eosinophil. (B) The photomicrograph is overexposed to aid the detection of unstained cells (eg, monocyte, lymphocyte, erythrocytes).

Table 2. Localization of BPI in Primary Granules: Evidence Suggesting Membrane Association

	Myeloperoxidase	Lactoferrin	Elastase	BPI*
Percoll gradient†				
α Fraction	80 $\pm$ 8	4 $\pm$ 4	NT	91 $\pm$ 5
β Fraction	20 $\pm$ 8	96 $\pm$ 4	NT	9 $\pm$ 5
Release during degranulation‡	22 $\pm$ 3	56 $\pm$ 9	63 $\pm$ 9	7 $\pm$ 2
Extraction§ from granules during treatment with				
Salt	78 $\pm$ 3	80 $\pm$ 2	NT	25 $\pm$ 3
Weak acid	>95	NT	>95	28–40

*Measured by radioimmunoassay.

†Distribution of granule proteins in pooled α and β fractions expressed as a percentage of the total recovered in those fractions. Values shown represent the mean $\pm$ SEM of three separate determinations.

‡Percentage of protein recovered in extracellular medium after treatment of neutrophils with cytochalasin B plus f-Met-Leu-Phe (mean $\pm$ SEM, $n = 3$). The spontaneous release from untreated cells (<5%) is subtracted. The total protein recovery (treated v untreated cells) is greater than 80%.

§Percentage of protein recovered from the supernatant at 20,000 g for 20 minutes after treatment of the mixed granule-rich fraction with salt (0.15 or 1.0 mol/L NaCl $\pm$ 20 mmol/L Hepes, pH 7.3; mean $\pm$ SEM; $n \geq 4$) or weak acid (0.2 mol/L sodium acetate/acetic acid, pH 4.0; range of two determinations).

neutrophil lineage and is present only in myeloid cells that contain primary granules, we examined several leukemic and nonleukemic human cell lines for BPI. Only cell lines with promyelocytelike cells (HL-60²⁹ and, to a lesser extent, KG-1²⁹) contained BPI as judged by immunofluorescence as well as by radioimmunoassay and bioassay of the cell extracts (Table 3). No BPI was detected in myeloid cell lines that do not mature beyond the myeloblast stage or in cells of other lineages.

Even in the HL-60 cell line (passage 30 to 40) with its predominance of promyelocytelike cells rich in myeloperoxidase^{29,34} (Table 4), only relatively few cells are BPI-positive, and the overall BPI content is low compared with mature neutrophils (either of normal or leukemic origin) (Table 3). Brightly staining BPI-positive cells are significantly enriched in the small subpopulation (about 15%)³⁵ of HL-60 cells that form rosettes with IgG-sensitized sheep erythrocytes (ie, Fc receptor-positive) and reciprocally diminished in the Fc receptor-negative cells (Table 4). More striking is the

almost mutually exclusive cellular localization of BPI and a cell surface antigen associated with cellular proliferative capacity that is recognized by monoclonal antibody Y201¹⁸ (Table 4, Fig 4A and B). As judged by double immunofluorescence, nearly all (88% $\pm$ 3%) BPI-positive cells are Y201-negative, which represents the less proliferative subpopulation (about 20% to 35%) of HL-60 cells.¹⁸ In contrast, Y201-positive and Y201-negative cells show nearly identical staining for myeloperoxidase (Table 4); only 26% $\pm$ 5% of the myeloperoxidase-positive HL-60 cells are Y201-negative.

Because the absence of the Y201 surface antigen and the presence of Fc receptors might reflect a more mature phenotype of the BPI-positive cells, we examined the effect of further differentiation in vitro on the BPI content of HL-60 cells. Treatment of HL-60 cells for four to six days with either retinoic acid (10^{-6} mol/L) or dimethyl sulfoxide (DMSO) (1.3%) induces differentiation in a granulocytic (neutrophilic) direction^{36,37} and increased the fraction of BPI-positive cells by up to twofold and the overall cellular content of BPI by 50% (Table 5). In contrast, during the same treatment, the myeloperoxidase content of HL-60 cells declines by two- to three-fold (Table 5).^{34,38} The increase in BPI content is restricted to granulocytic (neutrophilic) differentiation; treatment with either phorbol myristate acetate (10^{-8} mol/L) or a T cell-derived differentiation-inducing factor³⁹ induces monocyte/macrophage-like cellular characteristics^{39,40} and a reduction in the BPI content as judged by immunofluorescence (Table 5). Incubation of HL-60 cells in slightly alkaline medium induces eosinophilic differentiation⁴¹ and also a reduction in BPI-specific immunofluorescent staining (not shown).

DISCUSSION

As judged by immunofluorescence of intact cells and radioimmunoassay and bioassay of cell extracts, BPI occurs only in neutrophils and its precursors in peripheral blood or bone marrow and in myeloid (leukemic) cell lines. Along with other antimicrobial proteins and peptides of the neutrophil,^{4,5,42} BPI is located in the primary granules. BPI differs from other primary granule proteins in that it remains sedimentable under conditions that result in solubilization/release of proteins such as elastase and myeloperoxidase (Table 2). The highly basic BPI also exhibits hydrophobic properties. These are revealed in its interaction with target

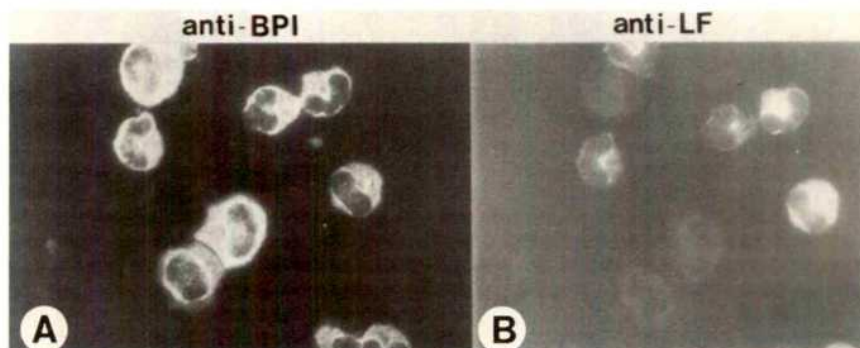


Fig 3. Double immunofluorescent staining of BPI (A) and lactoferrin (LF) (B) in human bone marrow cells.

Table 3. BPI Content of Various Human Cell Lines

Cell Line	Main Cellular Characteristics	Immunofluorescence (Percent Strong Fluorescence)	BPI Content* ($\mu\text{g}/10^8$ Cells)
Leukemic			
HL-60	Promyelocytes ^{29†}	14–23‡	14 $\pm$ 2
KG-1	Myeloblasts (promyelocytes) ²⁹	$\pm$ §	3–5
KG-1a	Myeloblasts ³⁰	0	NT
U-937	Monoblasts ³¹	0	NT
K 562	Early myeloid/erythroid blasts ²⁹	0	<0.5
MOLT-4, PS-2, EO-1	T lymphoblasts ³²	0	NT
Nonleukemic			
HeLa	Fibroblast	0	NT
HT 29	Colon adenocarcinoma	0	NT
A 673	Rhabdomyosarcoma	0	NT
SK-N-SH	Neuroblastoma ³³	0	NT
Mature neutrophils			
Normal		100	65 $\pm$ 6
CML		100	70 $\pm$ 5

Abbreviation: CML, chronic myeloid leukemia.

*Measured by radioimmunoassay and bioassay of crude cell extracts.

†References indicated within parentheses.

‡Range of seven determinations.

§Less than 20% of the cells are weakly positive.

||The BPI content of normal neutrophils is taken from Table 1; the BPI content of mature neutrophils of CML patients is taken from reference 6.

Table 4. Surface Marker Characteristics of BPI-Positive and Myeloperoxidase-Positive Subpopulations of HL-60 Cells

HL-60 (Sub)population	Immunofluorescent-Positive Cells (%) [*]	
	BPI	MPO
Total	18 $\pm$ 2	77 $\pm$ 5
Rosette-forming (FcR ⁺)†	58 $\pm$ 6	NT
FcR [−]	9 $\pm$ 2	NT
Y201 ⁺ ‡	3 $\pm$ 1	79 $\pm$ 5
Y201 [−]	77 $\pm$ 4	69 $\pm$ 3

The number of experiments is three for every subpopulation.

Abbreviation: MPO, myeloperoxidase.

*Percentage of brightly stained cells, mean $\pm$ SEM.

†Cells forming rosettes with IgG-sensitized sheep erythrocytes (ie, Fc receptor [FcR]-positive).

‡Cells staining (immunofluorescence) after incubation with the monoclonal antibody Y201 (74% $\pm$ 5% of the HL-60 cells, $n = 3$).

bacteria⁴³ and also with the synthetic hydrophobic matrices including phenyl-Sepharose and reverse-phase columns (unpublished observations), thus distinguishing BPI from other highly cationic granule proteins. The combination of strong positive charge and lipophilic properties may account for the firm association of BPI with the granule membrane.

Treatment of isolated granules with salt and/or weak acid reproducibly extracts 25% to 40% of the granule-associated BPI (Table 2). Strong acid (0.16 N H₂SO₄) is needed to solubilize most of the BPI.⁶ Whether the differential solubilization of BPI reflects heterogeneity in the BPI molecules or in their association with granule constituents^{44,45} and hence in their bioavailability for antibacterial action has not yet been determined. The localization of BPI in primary granules and in association with their membranes may favor the retention of this bactericidal agent within phagolysosomes⁴² and, consequently, its intracellular action against ingested bacteria.^{8,9}

Recent studies of the biosynthesis of myeloperoxidase and lactoferrin during myeloid differentiation have suggested that these granule proteins are produced during the limited time frame when granules are formed.^{46–48} The localization of BPI in primary granules implies therefore that BPI is synthesized at or around the promyelocyte stage²⁸ and before the synthesis of secondary granule proteins such as lactoferrin. It is fitting, therefore, that BPI⁺/lactoferrin[−] precursor cells in bone marrow stain most intensely for BPI (Fig 3) because it would be expected that the accumulation of BPI is limited to the period of assembly of the primary granules.

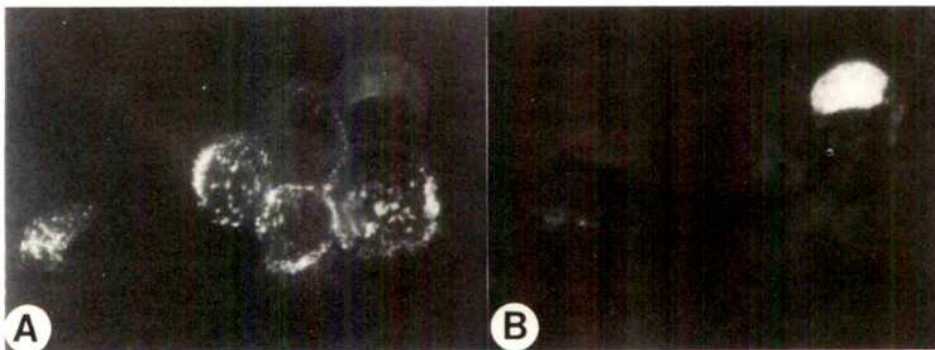


Fig 4. Double immunofluorescent staining of the proliferation-associated cell surface antigen (Y201) (A) and BPI (B) in HL-60 cells.

Table 5. Effect of Various Differentiation-Inducing Agents on the BPI Content of HL-60 Cells

Agent*	Differentiation†	Immunofluorescence (Percent Strong Fluorescence)	RIA ($\mu\text{g}/\text{mg}$ Cell Protein)
—	—	18 $\pm$ 1	1.2 $\pm$ 0.2
Retinoic acid (10^{-6} mol/L)	Granulocytic (63–88)	30	NT
DMSO (1.3%)‡	Granulocytic (60–84)	35 $\pm$ 2	1.8 $\pm$ 0.2
PMA (10^{-8} mol/L)	Monocytic (46–75)	8 $\pm$ 2	NT
DIF	Monocytic (39–65)	9	NT

Abbreviations: PMA, phorbol myristate acetate; DIF, differentiation inducing factor.

*HL-60 cells ($2 \times 10^5/\text{mL}$) were incubated for four days in medium containing the indicated supplement. After washing the cells in unsupplemented medium, the BPI content was measured. Results shown represent the mean ($\pm$ SEM, where indicated) of two or more independent determinations.

†Differentiation induced during treatment; numbers in parenthesis indicate the percentage of cells (range) displaying differentiated characteristics (see Materials and Methods).

‡At four days, cell densities in untreated and DMSO-treated cultures were identical ($10^6/\text{mL}$). DMSO treatment caused a reduction in the cellular content of myeloperoxidase: immunofluorescence, reduced from 81% to 37% bright staining; RIA, reduced from 29 to 12 $\mu\text{g}/\text{mg}$ cell protein.

with dilution of their contents during subsequent cell divisions.^{28,42} The fact that earlier myeloid precursors (myeloblasts) either in culture or in blood of patients with myeloid leukemia in blast crisis do not contain detectable BPI also fits the prediction.

Whether or not all primary granule proteins are synthesized at the same time is unknown. Some subcellular fractionation studies have revealed heterogeneity within putative primary granule fractions.^{44,45,49} HL-60 cells are rich in myeloperoxidase but contain relatively little BPI and also subnormal amounts of other primary granule proteins.^{34,38} The enrichment of BPI in an apparently more mature subpopulation (FcR + Y201–,^{18,50,51} Table 4) of uninduced cells together with the increase in the BPI content and decrease in the myeloperoxidase content that accompany further differentiation of HL-60 cells (induced by DMSO) may mean that in these cells synthesis of BPI follows that of myeloperoxidase. Such dissociation may be an abnormal feature of this leukemic cell line. However, these observations may also represent normal aspects of the biogenesis of primary granules. Because myeloperoxidase, unlike BPI, is present in monocytes as well as neutrophils,⁵² synthesis of myeloperoxidase may commence in a precursor cell capable of following either the neutrophilic or the monocytic paths,^{53,54} whereas synthesis of a neutrophil-specific protein such as BPI would not begin until the cell becomes committed to the neutrophilic lineage. The majority of HL-60 cells,

which are capable of differentiating in either a granulocytic or monocytic direction,^{53–55} may represent cells just before commitment when the dissociation of the accumulation of myeloperoxidase and BPI is clearly manifested. Because of the uncertainties concerning the fidelity of differentiation of malignant cells in culture, verification of these concepts must await similar studies on normal bone marrow cells.

That production of BPI is apparently limited to cells of the neutrophil lineage may reflect both the strict target cell specificity of this antibacterial protein^{3,6,7} and the predominant role of the neutrophil in host defense against BPI-sensitive gram-negative bacterial species.^{3,56} A comparison of the structural and functional properties of BPI isolated from human and rabbit polymorphonuclear leukocytes has shown that BPI is a highly conserved cytotoxic protein,^{3,6,7,57} which is consistent with the view that its exclusive production by the highly specialized neutrophil signifies an important antibacterial function.

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Dipyridamole Stimulates Urokinase Production and Suppresses Procoagulant Activity of Rabbit Alveolar Macrophages: A Possible Mechanism of Antithrombotic Action

By Jeffrey D. Hasday and Robert G. Sitrin

Dipyridamole, an inhibitor of platelet aggregation, has been shown to have beneficial effects in disorders characterized by extravascular fibrin deposition. Mononuclear phagocytes are present in extravascular sites and are capable of expressing both plasminogen activator and procoagulant activities, which suggests these cells play a central role in extravascular fibrin turnover. We therefore sought to determine whether dipyridamole affects the expression of plasminogen activator and procoagulant activities by rabbit alveolar macrophages cultured *in vitro*. We found that dipyridamole (10 to 100 $\mu\text{mol/L}$) caused increases in both cell-associated and released plasminogen activator activity, which reached levels of 240% ($P < .05$) and 543% ($P < .01$) of controls, respectively. In contrast, dipyridamole decreased the cell-associated procoagulant activity of alveolar macrophages to as little as 21.3% of controls ($P < .01$). Similar effects were seen in cells cotreated with lymphokines. The procoagulant activity expressed by

these cells functioned as a tissue thromboplastin. The plasminogen activator of control and treated cells was a urokinase as determined by molecular weight characteristics (50 kilodaltons) and by antibody neutralization profiles using polyclonal antibodies against human urokinase and tissue plasminogen activator. These effects of dipyridamole could not be duplicated by structurally dissimilar agents sharing some of the pharmacological actions of dipyridamole; however, two pyrimidopyrimidine compounds structurally similar to dipyridamole effectively mimicked the effects on both procoagulant and plasminogen activator activities. We conclude that dipyridamole may have antithrombotic effects by directly modulating the role of mononuclear phagocytes in fibrin turnover. Thus, dipyridamole may be useful in situations where extravascular fibrin deposition is important to the pathogenesis of tissue injury and repair.

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DIPYRIDAMOLE is a pyrimidopyrimidine compound widely used in treating thrombotic disease because of its actions as a platelet antiaggregant and vasodilator.¹⁻³ In addition, dipyridamole has been used successfully either singly or in combined therapy for glomerulonephritis^{4,5} and in animal models of glomerulonephritis and disseminated intravascular coagulation.^{6,7} These diseases are characterized histologically by extravascular fibrin deposition as well as intravascular platelet-fibrin thrombi.^{8,9} However, it is not clear how dipyridamole may also have its antithrombotic effects in the extravascular space where platelet aggregation and altered blood flow may not be the prime determinants of fibrin deposition.

Mononuclear phagocytes express procoagulant activity mainly by producing a tissue thromboplastin that activates the extrinsic coagulation pathway.^{10,11} These cells may also contribute to fibrinolysis by producing a plasminogen activator¹² or plasminogen-independent enzymes.^{13,14} We have previously demonstrated that rabbit alveolar macrophages produce both tissue thromboplastin and plasminogen activator activities.^{15,16} Since mononuclear phagocytes are often abun-

dant at sites of inflammation, they may be important cellular mediators of tissue deposition and clearance of fibrin. Therefore, in this study we sought to determine whether dipyridamole can directly affect macrophage expression of procoagulant or plasminogen activator activities. Such an effect would offer a potential explanation for the modulation by dipyridamole of extravascular fibrin turnover in inflammatory foci.

MATERIALS AND METHODS

Animals. Normal male New Zealand white rabbits (1.5 to 2 kg) were given routine care in the Unit for Laboratory Animal Medicine, University of Michigan Medical Center.

Bronchoalveolar lavage. Rabbits were sacrificed by intravenous injection of pentobarbital and exsanguinated prior to lavage. The trachea was cannulated, and the lungs were lavaged with 35-mL aliquots of Dulbecco's calcium and magnesium-free phosphate-buffered saline at 37 °C (PBS; GIBCO, Grand Island, NY) until a return of 300 mL was obtained. Cells were collected by centrifugation and resuspended in 110 mmol/L Tris buffer in 100 mmol/L NaCl (pH 7.0) for two minutes to lyse contaminating erythrocytes. The cells were then washed three times in serum-free culture medium consisting of RPMI 1640 (GIBCO) containing penicillin (100 U/mL), streptomycin (100 $\mu\text{g/mL}$), gentamicin (100 $\mu\text{g/mL}$), polymyxin B (100 U/mL), and lactalbumin hydrolysate (0.2%; GIBCO) and counted with a hemocytometer.

Preparation of additives. Dipyridamole (Sigma Chemical Co, St Louis) and related additives were freshly dissolved in 95% ethyl alcohol to 10 mmol/L and further diluted in culture medium. The final concentration of ethyl alcohol in cell cultures did not exceed 1% and was found to alter neither cell viability nor expression of procoagulant and plasminogen activator activities (data not shown).

Lymphocyte-conditioned medium (LCM) was used as a crude source of lymphokines. Lymphocyte suspensions were prepared from paraortic lymph nodes as previously described.^{16,17} The cell suspensions (>95% lymphocytes) were incubated for 48 hours at 5×10^6 cells/mL in medium containing concanavalin A bound to Sepharose 4B beads (5 $\mu\text{g/mL}$ ConA, Pharmacia Fine Chemicals, Piscataway, NJ). The LCM was then centrifuged to remove cells and ConA-

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Sepharose, sterilized by filtration, and stored at -70°C . Preliminary data indicated that a 1:8 dilution of LCM was optimal, and one such preparation was used in these experiments.

Cell culture. One million cells were dispensed in 1 mL of culture medium and incubated in duplicate in sterile 12×75 -mm polypropylene tubes for 24 hours at 37°C in 5% CO_2 -enriched air. Dipyridamole and other additives were included in the culture medium for the entire incubation period as indicated. Following incubation, the cells were collected by centrifugation and resuspended in fresh medium. Cell suspensions and incubation media were stored at -20°C prior to assay.

Procoagulant assay. The cells were lysed by two cycles of freeze-thawing followed by sonication. Preliminary experiments showed that the majority of the procoagulant activity remained cell associated *in vitro* because only minimal activity was found in the culture supernatants. None of the interventions tested caused a significant release of procoagulant activity into the incubation medium, thus indicating that modulation of procoagulant activity was adequately reflected by changes in the lysate activity. Accordingly, only cell lysates were routinely measured for procoagulant activity.

Procoagulant activity was assayed using a one-stage coagulation assay.¹⁶ One hundred microliters of test material was mixed with an equal volume of 25 mmol/L CaCl_2 at 37°C . Citrated normal rabbit plasma (100 μL) was then added, and the time elapsed until fibrin formation was measured with an automated coagulation timer (BBL Fibrosystem, Cockeysville, MD). Coagulation times of duplicate assays were reproducible within 12% over the range of activities encountered in this study. Standard curves were derived from serial dilutions of macrophage lysate by using a log-log plot of lysate concentration and the resultant coagulation time.¹⁶ Like identical assays using rabbit brain tissue thromboplastin (Sigma), these standards were linear for coagulation times ranging approximately from 20 to 260 seconds. Coagulation times could then be converted to arbitrary units of procoagulant activity to calculate the percent change from baseline activity induced by the various interventions.

To determine the mechanism of action of the procoagulant, samples were tested in coagulation assays using plasmas congenitally deficient in factors VIII, VII, or X (George King Bio-Medical, Overland Park, KS). In addition, the samples were pretreated with phospholipase C (Sigma), 0.05 $\mu\text{g}/\text{mL}$ for 30 minutes at 37°C because inactivation of procoagulant activity by this enzyme is indicative of tissue thromboplastin activity.¹¹

Plasminogen activator assay. Incubation media and cell lysates were assayed for plasminogen activator activity using a modification of the ^{125}I -fibrin plate assay of Unkeless et al.¹² Fibrin plates were prepared by adding to 16-mm plastic culture wells 60 μg of purified human fibrinogen (KABI, Stockholm, Sweden) containing 2×10^5 cpm ^{125}I -labeled fibrinogen (Amersham Corp, Arlington Heights, IL) and drying the plates at 37°C . The wells were then incubated with 5% acid-treated fetal bovine serum (GIBCO) to convert the fibrinogen to fibrin and then washed thoroughly with 0.1 mol/L Tris buffer (pH 8.1) prior to use. Plasminogen was prepared from outdated human plasma by lysine-Sepharose affinity chromatography (Pharmacia) according to the method of Deutsch and Mertz¹⁸ and quantified by protein content.¹⁹ To inactivate contaminating plasmin, the plasminogen preparation was incubated with phenylmethane sulfonyl fluoride (1 mmol/L) for 16 hours at 4°C followed by extensive dialysis. The plasminogen concentration that produced the maximal fibrinolysis with a standard sample of plasminogen activator was used in all assays (2 to 6 $\mu\text{g}/\text{mL}$).

To assay for plasminogen activator activity, test materials were added in duplicate with either plasminogen or buffer to ^{125}I -fibrin-coated wells in a final volume of 1 mL. Following incubation for four hours at 37°C , an aliquot of incubation medium was then removed

and ^{125}I activity measured with a gamma counter. Control wells containing either plasminogen or buffer alone were also included in each assay. Plasminogen activator activity was calculated as the difference between ^{125}I release in wells containing test material with plasminogen and the largest control value. The net ^{125}I release was expressed as the percentage of maximal release achieved by trypsinizing the wells. The resultant percent lysis was translated into milli-Plough units (mPU) by using a standard curve generated from human urokinase (Calbiochem, La Jolla, CA). Plasminogen-independent fibrinolysis was negligible in all samples and accounted for less than 5% of the fibrinolysis seen in the presence of plasminogen.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Culture supernatants from alveolar macrophages ($10^6/\text{mL}$) incubated for 24 hours in standard medium or medium containing dipyridamole (100 $\mu\text{mol}/\text{L}$) were concentrated 40-fold by pressure dialysis (Amicon Ultrafiltration System, Diaflo $\times$ PM10 membrane, Amicon Corp, Lexington, MA). Concentrated supernatants and standard samples of human urokinase and tissue plasminogen activator (tPA; American Diagnostica, Greenwich, CT) were treated with 10% SDS/8 mol/L urea. The samples were then subjected to electrophoresis at 20 mA on $60 \times 100 \times 0.4$ -mm gels of 11% polyacrylamide with 10% SDS in a Laemmli buffer system under nonreducing conditions.²⁰

Fibrin-agar zymography. Fibrin-agar indicator plates were created by pouring agarose (1.25%) containing fibrinogen (2 mg/mL), thrombin (Sigma; 0.06 National Institutes of Health U/mL), and plasminogen (5 $\mu\text{g}/\text{mL}$) onto glass slides.²¹ Following completion of electrophoresis, the polyacrylamide gels were washed sequentially in Triton X-100 (2.5%) and water in preparation for assay on the fibrin-agar plates. The gels were then layered over the indicator plates and incubated at 37°C for six to eight hours. The indicator plates were then stained with amido black, and the plasminogen activator activity was located by identifying the zones of lysis on the indicator plates.

Antibody neutralization. The plasminogen activator activities of control and dipyridamole-treated culture supernatants were characterized by neutralization profiles with antibodies against human urokinase and tPA. Immunoglobulin from antihuman urokinase goat serum (generously provided by G. Murano, PhD; Department of Biologicals, Food and Drug Administration, Bethesda, MD) was purified by diethyl aminoethyl chromatography and ammonium sulfate precipitation.²² Goat antihuman tPA immunoglobulin prepared in like fashion was purchased from American Diagnostica. Antibody concentrations were determined by the protein content.¹⁹ Test samples and the two plasminogen activator standards adjusted to equivalent activity were preincubated with a range of concentrations of antibody prior to the measurement of plasminogen activator activity. In these experiments, the plasminogen activator activity was measured using the two-stage spectrophotometric method of Coleman and Green.²³ The percent inhibition by antibody was calculated using standard curves for plasminogen activator activity generated for each sample.

Statistical analysis. For all dose-response curves, multiple comparisons with control groups were performed using Dunnett's test applied to a one-way analysis of variance.²⁴ Single comparisons were made using the unpaired two-tailed Student's *t* test.²⁴

RESULTS

Cell culture. The cell preparations used in these experiments were consistently more than 95% alveolar macrophages as described previously.¹⁵ In all cases, cell viability was greater than 95% prior to incubation as determined by trypan blue exclusion. Following incubation for 24 hours in

medium alone, the viability was $90\% \pm 7\%$ (mean $\pm$ SD). The viability was not significantly reduced by dipyridamole in concentrations ranging from $1 \mu\text{mol/L}$ ($90.1\% \pm 9.4\%$) to $50 \mu\text{mol/L}$ ($80\% \pm 15\%$).

The procoagulant activity of cells cultured in medium alone remained low over 24 hours (108.6 ± 15 seconds). Since augmented expression of procoagulant activity by these cells is a consistent response to low concentrations of endotoxin,¹⁵ this served as an internal control to indicate that the cells were not being subjected to significant background stimulation by endotoxin in the culture system.

Effects of dipyridamole on alveolar macrophage plasminogen activator activity. In control cultures incubated in standard medium alone, the plasminogen activator activity of cell lysates was $448 \pm 101 \text{ mPU}/10^6$ cells; in addition, $462 \pm 111 \text{ mPU/mL}$ was present in the incubation medium (mean $\pm$ SEM). Dipyridamole stimulated marked increases in the plasminogen activator activity released into the medium, which reached a maximum of 543% of control cultures ($P < .01$; Fig 1). There was also a significant increase in the activity of cell lysates, which reached 240% of the controls ($P < .05$), thus indicating that dipyridamole augmented the total expression of plasminogen activator rather than simply stimulating the release of intracellular stores of the enzyme into the culture media. Plasminogen activator activity of maximally stimulated cells ($50 \mu\text{mol/L}$ dipyridamole) was $1,132 \pm 322 \text{ mPU}/10^6$ cells in lysates and $1,619 \pm 774 \text{ mPU/mL}$ in media, both significantly increased from control values ($P < .05$).

Dipyridamole had no direct effect on plasminogen activator activity when mixed with cell lysates, thereby indicating that the drug did not activate an existing proenzyme. Finally, stimulation of plasminogen activator production was completely blocked by cycloheximide ($5 \mu\text{g/mL}$), which indicated a requirement for protein synthesis (not shown).

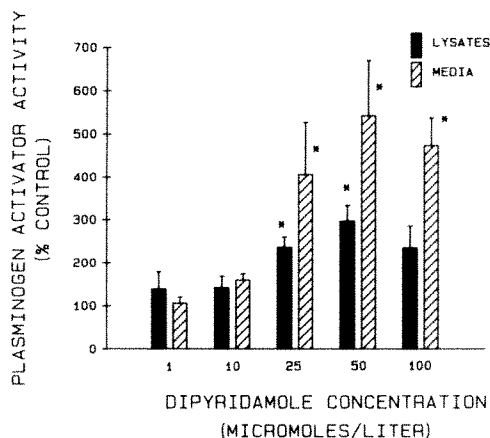


Fig 1. Effects of dipyridamole on the expression by rabbit alveolar macrophages of cell-associated and secreted plasminogen activator activities. Data are expressed as the percentage of values obtained from control cells maintained in parallel cultures without dipyridamole. The mean $\pm$ SEM of six to 14 experiments are shown. An asterisk indicates P value less than .01 (Dunnett's test). Dipyridamole caused a concentration-related stimulation in the plasminogen activator activity of both cell lysates and culture media.

Characterization of unstimulated and dipyridamole-stimulated plasminogen activator. Plasminogen activator activities of incubation media from control and dipyridamole-treated alveolar macrophages were significantly inhibited by antihuman urokinase antibody (Fig 2A). A human urokinase standard with equivalent activity was similarly inhibited by this antibody, but there was little cross-reactivity with human tPA. The anti-tPA antibody had only a slight neutralizing effect on the control media and none on media from dipyridamole-treated cells (Fig 2B). This was comparable to the effect of antibody on urokinase standard, whereas tPA was completely neutralized. These results indicate that the plasminogen activator of normal and dipyridamole-treated alveolar macrophages is a urokinase rather than a tPA.

SDS-PAGE analysis of incubation media from both control and dipyridamole-treated cells revealed a single band of plasminogen activator activity with a molecular weight of approximately 50 kilodaltons (kD) (Fig 3). A human urokinase standard demonstrated a major band of activity of 53 kD and a minor band of 32 kD, whereas human tPA produced a single band of 68 kD. Together with the antibody neutralization experiments, these results indicate that dipyridamole caused increased production of a single form of urokinase rather than stimulating production of a second protease with plasminogen activator activity.

Effects of dipyridamole on alveolar macrophage procoagulant activity. The procoagulant activities of cell lysates

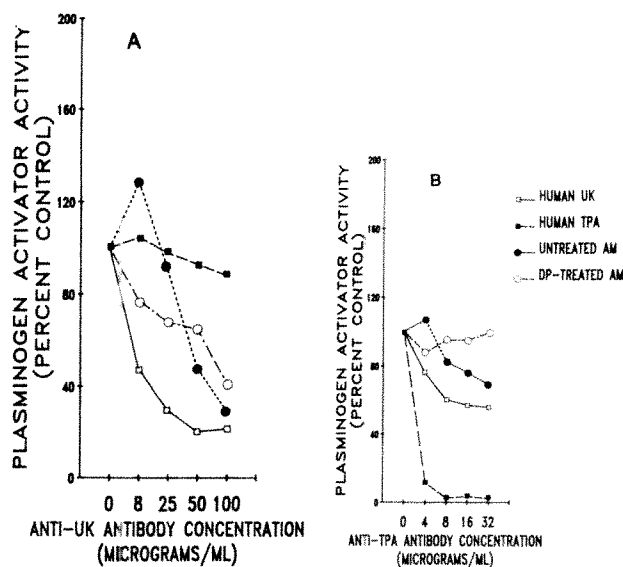


Fig 2. Characterization of plasminogen activator by neutralization with antiurokinase (anti-UK) (A) and anti-tPA (B) antibodies. Incubation media from normal and dipyridamole (DP)-treated alveolar macrophages were studied along with commercially prepared urokinase and tPA to determine the percent inhibition by each antibody (see Materials and Methods). The data are expressed as the mean percent inhibition of four experiments. The antiurokinase antibody effectively suppressed the plasminogen activator activity of the urokinase standard and both incubation media but not tPA (A). Likewise, the anti-tPA antibody effectively neutralized the tPA standard but neither urokinase nor the incubation media (B).

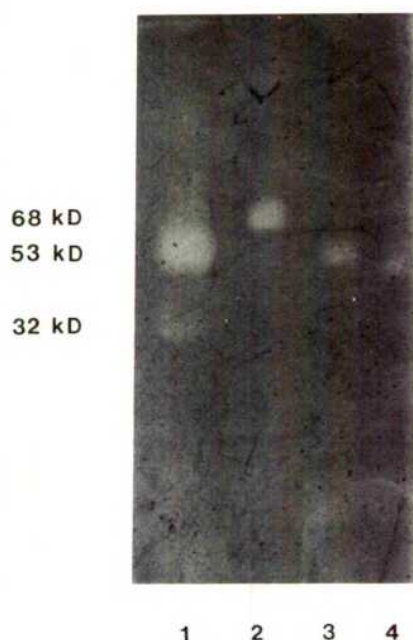


Fig 3. Molecular weight determination of rabbit alveolar macrophages plasminogen activator. Shown is the fibrin-agar indicator plate developed from an SDS-PAGE (see Materials and Methods). Human urokinase (lane 1) demonstrated a major band at 53 kD and a minor band at 32 kD, whereas human tPA produced a single band at 68 kD (lane 2). Incubation media from both dipyridamole-treated (lane 3) and untreated (lane 4) macrophages produced a single band of activity at 50 kD, virtually identical to the major band of human urokinase.

from control cultures were somewhat variable, but dipyridamole consistently and significantly decreased the expression of procoagulant activity in parallel cultures to as little as 21.3% of control values ($P < .01$; Fig 4A). The corresponding prolongation in coagulation time was also significant, increasing from 108.6 ± 15 seconds in controls to 190.1 ± 58.7 seconds in cells treated with $50 \mu\text{mol/L}$ dipyridamole ($P < .05$). To determine whether dipyridamole also suppressed the augmentation of procoagulant activity in response to an immunostimulant, alveolar macrophages were cocultured with dipyridamole and LCM. In these experiments, dipyridamole ($10 \mu\text{mol/L}$ and $100 \mu\text{mol/L}$) also decreased the procoagulant activity by as much as 83.3% (Fig 4B). The coagulation times were prolonged from 66.8 ± 10.7 seconds (LCM alone) to 96.5 ± 15.7 seconds ($10 \mu\text{mol/L}$, $P < .01$) and 171.3 ± 23.2 seconds ($100 \mu\text{mol/L}$, $P < .01$). Additional experiments verified that dipyridamole had similar suppressive effects on the procoagulant activity expressed by suspensions of intact cells (not shown).

Dipyridamole had no direct effect on the coagulation assay. Second, the loss of procoagulant activity in dipyridamole-treated cells was not affected by incorporating excess phospholipid (rabbit brain cephalin; Sigma) in the assay system, which suggests that the drug did not simply alter the phospholipid content of the lysates. Finally, in mixing experiments the addition of lysates of dipyridamole-treated cells lacking detectable procoagulant activity did not inhibit the activity of lysates of control cells, which indicates that

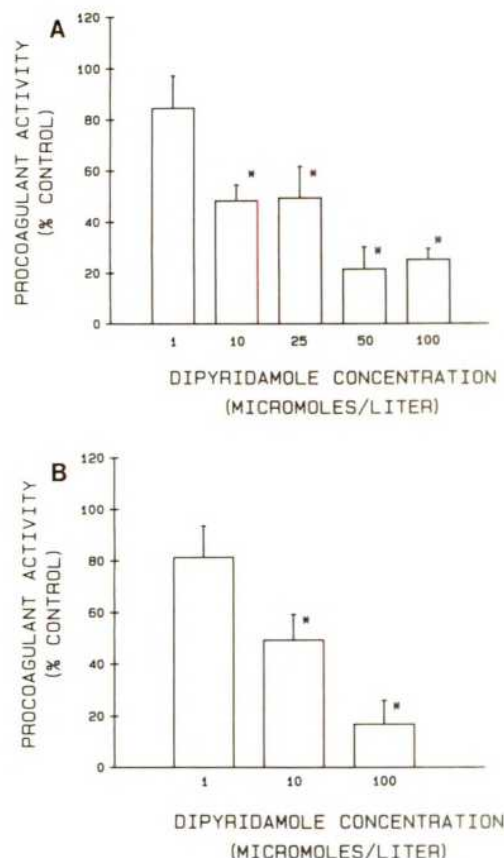


Fig 4. Effects of dipyridamole on the expression of procoagulant activity by unstimulated (A) and LCM-stimulated rabbit alveolar macrophages (B). Activities are expressed as the percentage of values obtained from control cells lacking dipyridamole; the mean $\pm$ SEM of six to 14 experiments are shown. An asterisk indicates a P value less than .01 (Dunnett's test). Dipyridamole caused a concentration-dependent suppression of procoagulant activity by both unstimulated and LCM-stimulated alveolar macrophages.

dipyridamole did not stimulate the expression of an inhibitor of procoagulant activity. The procoagulant activity of both untreated and dipyridamole-treated cell lysates functioned as tissue thromboplastin.¹⁶ This was demonstrated by a loss of procoagulant function in plasmas deficient in factors VII or X but not VIII (not shown). In addition, procoagulant activity was inactivated by pretreatment by phospholipase C, which inactivates tissue thromboplastin but not other coagulation factors.¹¹

Mechanism of action of dipyridamole. Experiments were performed to determine the mechanisms by which dipyridamole modulates expression of macrophage procoagulant and plasminogen activator activities. The effects of dipyridamole were (1) compared with those of structurally unrelated compounds sharing at least one of the known pharmacological actions of dipyridamole, (2) examined in the presence of agents that antagonize specific actions of dipyridamole, and (3) compared with those of structurally related pyrimidopyrimidines.

Dipyridamole is known to alter production and cellular responses to various arachidonic acid metabolites.²⁵ To deter-

mine whether dipyridamole acts through mechanisms involving arachidonic acid metabolism, alveolar macrophages were cotreated with dipyridamole, 100 $\mu\text{mol/L}$, and indomethacin, 1 $\mu\text{mol/L}$, a potent cyclooxygenase inhibitor. The addition of indomethacin did not alter the effects of dipyridamole on the expression of either procoagulant or plasminogen activator activities (Fig 5A). Further, we have demonstrated that neither exogenous arachidonic acid metabolites nor cyclooxygenase/lipoxygenase inhibitors could mimic the effects of dipyridamole on these macrophage functions (Hasday and Sitrin, manuscript submitted), thereby indicating that arachidonic acid metabolites are not directly involved in the action of dipyridamole.

Dipyridamole is known to inhibit the transport of adenosine across plasma membranes, which leads to extracellular accumulation of adenosine and increased stimulation of plasma membrane adenosine receptors.²⁶ To determine whether dipyridamole modulates procoagulant and plasminogen activator activities by blocking adenosine transport, we cotreated alveolar macrophages with dipyridamole and theophylline, 100 $\mu\text{mol/L}$, which blocks the adenosine receptor.²⁷ The addition of theophylline did not block the effects of dipyridamole (Fig 5B). Second, we compared cells treated with dipyridamole with those treated with nitrobenzylthioinosine (NBTI, 0.1 to 100 $\mu\text{mol/L}$), another inhibitor of adenosine transport.²⁶ NBTI did not mimic the effects of dipyridamole, but caused only moderate suppression of procoagulant activity, and had no effect on the plasminogen activator activity (Fig 5B). These results indicate that the action of dipyridamole is not based on the inhibition of adenosine transport across cell membranes.

Dipyridamole is known to inhibit cyclic adenosine monophosphate (AMP)-phosphodiesterase.²⁸ To determine whether this action pertains to its effects on procoagulant and plasminogen activator expression, we compared the

effects of dipyridamole with those of other phosphodiesterase inhibitors, papaverine (10 $\mu\text{mol/L}$) and methylisobutylxanthine (MIX, 100 $\mu\text{mol/L}$). In contrast to the effects of dipyridamole, papaverine significantly inhibited both plasminogen activator and procoagulant activities by 56.5% ($P < .01$) and 45% ($P < .05$), respectively (Fig 5C). MIX inhibited plasminogen activator activity by 69% ($P < .01$) but had no significant effect on procoagulant activity. These results indicate that inhibition of cyclic AMP phosphodiesterase cannot fully explain the actions of dipyridamole. The mechanism of action of these phosphodiesterase inhibitors is likewise unclear. We have recently shown that dibutyryl cyclic AMP suppresses plasminogen activator secretion but not cell-associated procoagulant activity, thus indicating that the effects of papaverine and MIX cannot be explained solely by a global increase in intracellular levels of cyclic AMP (Hasday and Sitrin, manuscript submitted).

Finally, experiments were performed to investigate whether the effects of dipyridamole were shared by other pyrimidopyrimidines. Alveolar macrophages were treated with *RXRA-85* (4-[1-oxidothiomorpholino]-8-[2-phenylethylthio]-2-piperizino-pyrimido/5, 4-D/pyrimidine) and *RE-102* (3,6-dimorpholino-8[bis-(2-hydroxypropyl)-amino-2-phenyl-pyrimido-(5,4-B)pyrize], two structural analogues of dipyridamole kindly provided by the Boehringer-Ingelheim Co (FR Germany). The effects of optimal concentrations of these compounds on the expression of procoagulant and plasminogen activator activities are shown in Fig 6. Both *RXRA-85* (10 $\mu\text{mol/L}$) and *RE-102* (100 $\mu\text{mol/L}$) significantly inhibited expression of procoagulant activity (69.7% and 92.3% respectively, $P < 0.01$). In addition, the release of plasminogen activator into culture media was markedly stimulated (325% and 486% respectively, $P < 0.01$), effectively duplicating the effects of dipyridamole.

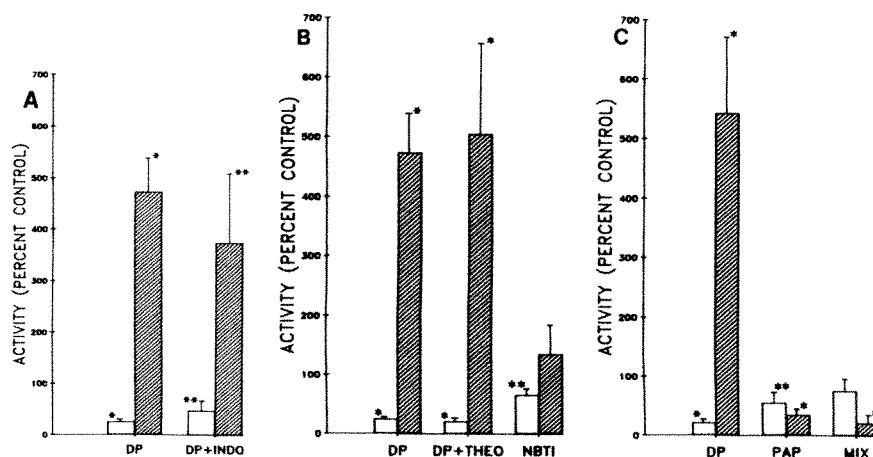


Fig 5. Investigation of the mechanism by which alveolar macrophages modulate procoagulant and plasminogen activator activities. Data are expressed as in Fig 1. (A) The actions of dipyridamole (DP, 100 $\mu\text{mol/L}$) were unaffected by cotreatment with indomethacin (INDO, 1 $\mu\text{mol/L}$). (B) The effects of dipyridamole were not altered by cotreatment with theophylline (THEO, 100 $\mu\text{mol/L}$), which blocks plasma membrane adenosine receptors. Likewise, these effects were not duplicated by treating macrophages with NBTI (10 $\mu\text{mol/L}$), which also blocks adenosine transport across plasma membranes. (C) The effects of dipyridamole could not be duplicated by two other phosphodiesterase inhibitors, papaverine (PAP, 10 $\mu\text{mol/L}$) and methylisobutylxanthine (MIX, 100 $\mu\text{mol/L}$). □, Procoagulant; ▨, PLG activator.

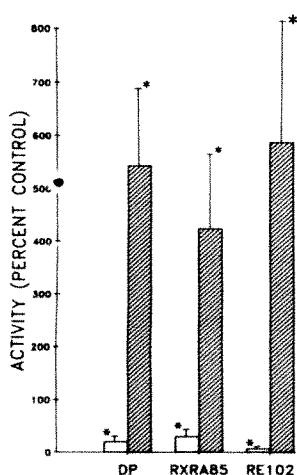


Fig 6. Effects of structural analogues of dipyridamole on alveolar macrophage procoagulant and plasminogen activator activities. Optimal concentrations of the agents are compared and data are expressed as in Fig 1. The effects of dipyridamole (DP, 50 μ mol/L) were duplicated by RXRA-85 (10 μ mol/L) and RE-102 (100 μ mol/L). $\square$, Procoagulant; ▨ , PLG activator.

DISCUSSION

The close association between inflammatory reactions and activation of the coagulation and fibrinolytic systems is well recognized.^{8,9,29-31} The clinical manifestations of these interrelationships include disseminated intravascular coagulation and localized formation of intravascular fibrin-platelet thrombi.^{29,31} In addition, fibrin deposition outside the vascular space is prominent in many inflammatory states such as cutaneous hypersensitivity, glomerulonephritis, and acute lung injury.^{8,30,31} Various methods of anticoagulation have suppressive effects on cutaneous hypersensitivity and glomerulonephritis, which suggests that products of coagulation may intensify inflammation.^{32,33} In vitro experiments have demonstrated that fibrinogen-related products are leukocyte chemotaxins, impair macrophage mobility, and induce microvascular permeability.³⁴⁻³⁶

Although the factors controlling extravascular fibrin turnover are not well elucidated, there is considerable evidence to suggest that mononuclear phagocytes are central to this process. These cells can directly promote coagulation by elaborating tissue thromboplastin and other coagulation factors as well as mediating fibrinolysis by producing plasminogen activator or fibrinolytic enzymes.¹⁰⁻¹⁷ Both tissue thromboplastin and plasminogen activator activities can be stimulated in vitro by many stimuli relevant to inflammation including lymphokines and bacterial lipopolysaccharides.¹⁰⁻¹⁷ Recent studies have suggested that macrophage-derived procoagulant activity contributes to local fibrin deposition in experimental glomerulonephritis, thereby supporting the potential importance of these activities in vivo.³⁷ Further, Chapman et al have demonstrated abnormal expression of procoagulant and plasminogen activator activities by alveolar macrophages from some patients with interstitial lung disease.³⁸ Currently, there are few options for pharmacologically manipulating the effect of macrophages on fibrin deposition and clearance. Glucocorticosteroids, indomethacin, Coumadin, and prostaglandin E modify expression of procoagulant or plasminogen activator activities in vitro, but no other agents potentially applicable to in vivo use have been identified.^{32,39-41}

Several studies have demonstrated beneficial effects of dipyridamole in glomerulonephritis and attributed these to its actions as an inhibitor of platelet function.⁴⁻⁶ Since the crescentic fibrin deposition seen in an animal model appears to be caused by infiltrating macrophages,³⁷ we sought to determine whether dipyridamole directly modulates the expression of procoagulant or fibrinolytic activities of mononuclear phagocytes. Using normal rabbit alveolar macrophages, we found that dipyridamole had profound effects on the expression of both activities in vitro because expression of tissue thromboplastin activity was suppressed by over 80% whereas the concurrent release of plasminogen activator was increased by more than fivefold. The suppression of tissue thromboplastin activity occurred by decreased cellular expression of this factor rather than by stimulating production of an inhibitory protein or by directly affecting the extrinsic coagulation pathway. Likewise, dipyridamole directly stimulated cellular production of plasminogen activator rather than causing the release of the enzyme from intracellular stores or by activating a preexisting proenzyme. The plasminogen activator was found to be a single protein with molecular weight and immunologic characteristics of urokinase. The mechanisms underlying these actions of dipyridamole remain undefined. We determined that structurally unrelated compounds sharing at least one of the well-recognized pharmacological actions of dipyridamole, namely cyclic AMP-phosphodiesterase inhibition,²⁸ blockade of purine transport across plasma membranes,²⁶ and alteration of arachidonic acid metabolism²⁵ could not reproduce both these effects. However, two other structurally related pyrimidopyrimidines were roughly equipotent in their effects on both tissue thromboplastin and urokinase activities.

Further studies are certainly necessary to determine whether dipyridamole has comparable effects on mononuclear phagocytes in vivo. However, the potential biologic relevance of the findings reported here is supported by the observation that dipyridamole affected both activities in vitro at concentrations ranging from 10 to 25 μ mol/L. Plasma concentrations in excess of 10 μ mol/L can be achieved in some human subjects with the peak effect of a single 75-mg dose, and considerably higher doses have been given safely.^{4,42} It also remains to be seen whether prolonged administration in vivo can achieve comparable effects even with lower ambient concentrations of this drug.

The results of this study suggest several important areas for future investigations. First, it will be necessary to explore the possibility that dipyridamole and related compounds can be therapeutic in inflammatory disorders by preventing fibrin from accumulating in tissues. These studies should also better define the importance of macrophage-directed fibrin turnover in the pathogenesis of inflammation and healing. One potential advantage to this approach is that fibrin deposition may be selectively suppressed in injured tissue where macrophages have accumulated without inducing a severe generalized hemostatic defect. Second, the possibility is raised that dipyridamole may have other striking effects on macrophage functions beyond the two activi-

ties reported here, thereby suggesting that the pyrimidopyrimidines represent a new class of pharmacological agents for modulating mononuclear phagocyte function in inflammatory diseases in vivo. Further supporting this hypothesis are limited studies demonstrating that dipyridamole inhibits lymphoproliferation and stimulates interferon secretion by leukocytes in vitro.^{43,44} Such investigations may also provide important new information on the mechanisms underlying the pharmacological actions of pyrimidopyrimidines as well as the expression of macrophage effector functions. Finally, these actions of dipyridamole may have important implications for investigations of atherosclerotic diseases. Prior studies have demonstrated that macrophages infiltrate atheromas and release factors that may contribute to their progression.⁴⁵ Moreover, dipyridamole has inhibited plaque formation in experimental atherosclerosis.⁴⁶ Given the current evidence for the role of thrombus formation in coronary artery occlusion and the widespread interest in thrombolytic

and anticoagulant therapies for management of myocardial ischemia,^{47,48} the possibility for pharmacologically suppressing some of the thrombogenic activity in atheromas while concurrently increasing local fibrinolysis certainly merits further investigation.

In summary, we have demonstrated that dipyridamole causes alveolar macrophages to decrease the expression of tissue thromboplastin activity while concurrently stimulating the production and release of urokinase. These combined actions suggest that dipyridamole may effectively prevent fibrin accumulation at sites of inflammation through its direct effects of mononuclear phagocytes.

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Synergistic Action of Two Murine Monoclonal Antibodies That Inhibit ADP-Induced Platelet Aggregation Without Blocking Fibrinogen Binding

By Peter J. Newman, Rodger P. McEver, Mary P. Doers, and Thomas J. Kunicki

We have used two murine monoclonal antibodies, each directed against one component of the human platelet membrane glycoprotein (GP) IIb-IIIa complex, to examine further the molecular requirements for fibrinogen binding to the platelet surface and subsequent platelet-platelet cohesion (aggregation). Although neither AP3, which is directed against GPIIb, nor Tab, which is specific for GPIIb, were individually able to inhibit adenosine diphosphate (ADP)-induced fibrinogen binding, platelet aggregation, or secretion, the combination of AP3 and Tab completely abolished platelet aggregation and the release reaction. Unexpectedly, this synergistic inhibition of platelet-platelet cohesion occurred in the presence of apparently normal fibrinogen binding. Both the number of fibrinogen molecules bound and the dissociation constant for fibrinogen binding remained essentially unchanged in the presence of

these two antibodies. Inhibition of aggregation was dependent upon the divalency of both AP3 and Tab because substitution of Fab fragments of either antibody for the intact IgG resulted in a complete restoration of both aggregation and secretion. In contrast to ADP induction, thrombin-activated platelets neither aggregated nor bound fibrinogen in the presence of AP3 plus Tab but were fully capable of secretion, which illustrated the multiple mechanisms by which the platelet surface can respond to different agonists. These data demonstrate that fibrinogen binding to the platelet surface alone is not sufficient to support platelet-platelet cohesion and that an additional post-fibrinogen-binding event(s) that is inhibitable by these two monoclonal antibodies may be required.

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FOLLOWING BLOOD VESSEL injury, platelets rapidly adhere to the exposed subendothelium, release the contents of their granules, and form platelet aggregates that are responsible for the primary arrest of bleeding. Platelet plasma membrane glycoproteins mediate many of these events and serve as receptors for several physiological agonists and cofactors important in this hemostatic process including thrombin, collagen, von Willebrand factor (vWf) and fibrinogen.¹⁻³ There is considerable evidence that platelet-platelet cohesion (aggregation) is mediated by the binding of fibrinogen, an elongated, symmetrical molecule of 340,000 daltons,⁴ to adjacent platelets. Studies in a number of laboratories have demonstrated that adenosine diphosphate (ADP)^{5,6} thrombin,^{7,8} epinephrine,^{5,9} and certain stable prostaglandin analogues¹⁰ induce platelet cohesion by exposing fibrinogen receptors on the platelet surface. Fibrinogen then binds to activated platelets via a heterodimer receptor complex composed of plasma membrane glycoproteins (GP) GPIIb and IIIa (reduced molecular weights of 130,000 and 100,000 daltons, respectively). Evidence for this is derived, in part, from studies on individuals with the congenital bleeding

disorder Glanzmann's thrombasthenia. The platelets of these patients are deficient in these two membrane glycoproteins,^{11,12} and consequently their platelets fail both to bind fibrinogen and to aggregate normally.^{5,13,14} In addition, the binding to normal platelets of murine monoclonal antibodies specific for the intact GPIIb-IIIa complex induces a thrombasthenic condition by inhibiting both fibrinogen binding and subsequent aggregation.¹⁵⁻¹⁸ Together, these data have been taken to indicate that platelet aggregation is an inevitable consequence of fibrinogen binding to its platelet surface receptor.

We have previously reported the production and characterization of monoclonal antibodies specific for components of the platelet fibrinogen receptor: Tab, which is specific for GPIIb¹⁶; AP3, which is specific for GPIIa¹⁹; and AP2, a monoclonal antibody reactive only with the intact GPIIb-IIIa complex.¹⁵ When bound to the platelet surface, AP2 blocks both fibrinogen binding and platelet aggregation induced by ADP, thrombin, arachidonic acid, or collagen. Unlike AP2, neither Tab nor AP3 are able to block the binding of fibrinogen to the platelet surface or inhibit platelet aggregation, presumably because the epitopes recognized by these two antibodies are not close enough to the fibrinogen binding site to inhibit its interaction with the GPIIb-IIIa complex. Though only Tab and AP3 react with both dissociated as well as complexed GPIIb and GPIIa, all three antibodies react with 40,000 to 50,000 sites at saturation on the platelet surface, thus supporting the concept that GPIIb and GPIIa normally exist complexed in a 1:1 stoichiometry in the plasma membrane of intact, nonactivated platelets.¹⁹ In this report, we describe a synergistic effect of AP3 and Tab on ADP-induced platelet aggregation that provides new insights into the molecular requirements for fibrinogen binding and the process of platelet aggregation.

MATERIALS AND METHODS

Preparation of washed platelet suspensions. Human platelets were isolated from whole blood using a modification of the method of Patscheke.²⁰ Briefly, 43 mL of venous blood obtained from healthy volunteer donors was anticoagulated with 7 mL of acid citrate

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dextrose (ACD, National Institutes of Health formula A). Following the preparation of platelet-rich plasma (PRP), prostaglandin E_1 , (PGE_1) and apyrase (Sigma Chemical Co, St Louis; Grade III) were added to the platelets to final concentrations of 20 nmol/L and 25 μ g/mL, respectively. ACD was then added in the ratio of one part ACD to nine parts PRP to further acidify the PRP. Platelets were washed three times by differential centrifugation at 1,200 g in Wash buffer (103 mmol/L NaCl, 36 mmol/L citric acid, 5 mmol/L dextrose, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 0.35 mg/mL bovine serum albumin (BSA), 20 nmol/L PGE_1 , and 25 μ g/mL apyrase, pH 6.5) and resuspended into Resuspension buffer (137 mmol/L NaCl, 2 mmol/L KCl, 0.3 mmol/L NaH_2PO_4 , 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 5.5 mmol/L dextrose, 5 mmol/L HEPES, 12 mmol/L $NaHCO_3$, and 0.35 BSA, pH 7.4) before use. Both Wash and Resuspension buffers were used for a maximum of 1 week. Platelets prepared in this manner retained the ability to aggregate and bind fibrinogen for at least four hours.

Proteins. The purification and properties of the murine monoclonal antibodies AP1, AP2, AP3, and Tab have been previously described^{15,16,18,21} and are described in Table 1. AP1 is specific for human platelet membrane GPIIb, AP2 is specific for the GPIIb-IIIa complex, AP3 has specificity for only GPIIIa, and Tab is specific for only GPIIb. Monoclonal IgG used in these studies was purified by sequential ammonium sulfate fractionation and diethyl aminoethyl (DEAE)-cellulose chromatography or by caprylic acid precipitation as described previously.^{19,22} Fab fragments of AP3 and Tab were produced by digestion for five hours at 37 °C with immobilized papain (Pierce Chemical Co, Rockford IL) in a buffer containing 50 mmol/L NaH_2PO_4 , 5 mmol/L EDTA, and 20 mmol/L cysteine, pH 6.5. Reaction products were alkylated for 30 minutes in the dark with 15 mmol/L iodoacetamide and dialyzed into 0.1 mol/L NaH_2PO_4 , pH 8.0. Fab fragments were separated from Fc fragments and undigested IgGs by chromatography through protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and their purity assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under both reducing and nonreducing conditions. Chromatographically pure Peak 1 human fibrinogen, a kind gift from Dr D. Amrani, Mount Sinai Medical Center, Milwaukee, was shown to be free of contaminating vWf and fibronectin by both quantitative electroimmunoassay and the inability to support ristocetin-induced platelet aggregation. Purified fibrinogen was approximately 95% clottable prior to labeling and greater than 90% clottable following radioiodination and storage.

Radioiodination of proteins in solution. Fibrinogen was radioiodinated by reacting 5 mg of protein with 0.3 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston) for 15 minutes in the presence of insolubilized lactoperoxidase (Enzymobeads, Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions. Fifty to 100 μ g of each monoclonal antibody was labeled with 1 mCi of ^{125}I in a similar fashion. Following iodination, unbound ^{125}I was removed from the reaction mixture by gel filtration through Sephadex G-25 equilibrated in resuspension buffer. The specific activity of the fibrinogen was approximately 100 cpm/ng and approximately 1,000 cpm/ng for each of the monoclonal antibodies.

Radioiodinated antibodies were stored in small aliquots at -80 °C. Labeled fibrinogen was stored at 4 °C and used within 1 week.

Platelet aggregometry. Aggregometry was performed in a Bio-Data PAP-4 four-channel aggregometer (Bio-Data Corp, Horsham, PA) at 37 °C with a stirring rate of 1,200 rpm. ADP was used to induce aggregation at concentrations of 5, 10, and 20 μ mol/L. Thrombin and epinephrine were used at concentrations of 0.1 to 1.0 U/mL and 2.2 μ mol/L, respectively. The effects of monoclonal antibody binding on platelet aggregation were measured by preincubating washed platelets with the indicated concentrations of antibody for five minutes at 37 °C prior to the induction of aggregation. Lumiaggregometry was performed using a Chrono-Log Model 400 Lumiaggregometer. The luciferase-luciferin reagent was also from Chrono-Log Corp (Havertown, PA).

Quantitative fibrinogen binding analysis. Binding assays were performed in triplicate at room temperature (except where indicated) using washed platelets prepared as described. 1.5-milliliter polypropylene tubes precoat with 1 mL BSA dissolved in Resuspension buffer were used in all phases of the experiment. For the ADP-induced fibrinogen binding experiments, ^{125}I -fibrinogen was added at varying concentrations to washed platelets in Resuspension buffer such that the final platelet concentration was 1×10^8 /mL in a reaction volume of 200 μ L. Preliminary experiments (not shown) established that normal ADP-induced fibrinogen binding was saturable and required the presence of calcium. Monoclonal antibodies in the form of intact IgG or Fab fragments were added either prior to or simultaneous with the addition of fibrinogen and allowed to incubate with the platelets for five to ten minutes before the addition of ADP. Binding was initiated by addition of ADP to final concentrations ranging from 3.3 to 33 μ mol/L. The reaction was terminated five minutes later by centrifugation (12,000 g for three minutes) of a 75- μ L aliquot over 400 μ L of 15% sucrose dissolved in Resuspension buffer. After removing the supernatant, the centrifuge tube tips were amputated and the bound radioactivity determined in a gamma counter. Thrombin-induced fibrinogen binding was induced by incubating platelets that had been preincubated with antibody for ten minutes with 0.1 U/mL of α -thrombin (a gift of Dr Richard A. Marlar, The Blood Center of Southeastern Wisconsin, Milwaukee) for five minutes at 25 °C. Thrombin was inactivated by the addition of hirudin (0.2 U/mL) for five minutes, and then ^{125}I -fibrinogen was added to initiate binding. The reaction was terminated 15 minutes later by centrifugation and aspiration, as described above. In selected experiments, monoclonal antibodies or their Fab fragments were added following rather than prior to thrombin/hirudin activation of platelets. The bound radioactivity was pure fibrinogen, as determined by SDS-PAGE and autoradiographic analysis of the solubilized pellets. The total radioactivity was determined by counting an identical 75- μ L aliquot of each reaction mixture. Nonspecific binding was determined in parallel tubes containing 10 mmol/L EDTA, and this value was subtracted from the total bound counts per minute to yield specifically bound counts per minute. This value was shown to be comparable to nonspecific binding levels obtained in the presence of a 25-fold excess of unlabeled fibrinogen. Fibrinogen binding affinities and the maximum number of sites

Table 1. Murine Monoclonal Antibodies Specific for Human Platelet Membrane GPIIb and IIIa

Antibody	Specificity	No. Sites/Platelet	K_D (nmol/L)	Inhibition of Fibrinogen Binding	Inhibition of Platelet Aggregation	Reference
AP2	GPIIb-IIIa complex	54,000	0.64	Yes	Yes	15
AP3	GPIIIa	40,000	0.72	No	No	19
Tab	GPIIb	40,000	0.73	No	No	16
AP1	GPIIb	20-30,000	2.10	No	No	21

bound per platelet were calculated and analyzed using the weighted nonlinear least-squares curve fitting program LIGAND.²³ Objective statistical criteria (residual variance, F test) were used to evaluate the goodness of fit and to discriminate between models testing different classes of binding sites.

Measurement of β -thromboglobulin (β -TG) release. Platelets in PRP at a final concentration of 1×10^8 /mL were added to an aggregometer cuvette and incubated in an aggregometer at 37 °C for two minutes. Antibodies diluted in platelet-poor plasma (PPP) or PPP alone were added and incubated an additional ten minutes with stirring (1,000 rpm). Aggregation and release were induced by the addition of 20 μ mol/L ADP/2.2 μ mol/L epinephrine, and activation was allowed to proceed for 3.5 minutes, ie, the amount of time necessary to achieve maximal aggregation in those samples that were able to aggregate. PGE₁ was then added to the cuvette to a final concentration of 20 ng/mL to inhibit further activation and release that might occur as a result of subsequent handling. An 85- μ L aliquot was removed from the cuvette, and released β -TG was quantitated by using a standard radioimmunoassay kit (Amersham Corp, Arlington Heights, IL).

Competitive monoclonal antibody binding experiments. A quantity of 3.6 ng of radiolabeled monoclonal antibody was added to 6×10^6 platelets in the presence or absence of a 250-fold excess of the indicated unlabeled monoclonal antibody. This amount was predetermined to give the maximal inhibition of binding of an identical radioiodinated antibody. The volume was adjusted with PRP to 300 μ L to yield a final platelet concentration of 2×10^7 /mL. Both labeled and unlabeled antibodies were added to the platelets at the same time to ensure equal opportunity for binding. After a 30-minute incubation at room temperature, the aliquots were removed, and the bound radioactivity was determined as described. Nonspecific binding varied from 3% to 9% for each of the monoclonal antibodies.

RESULTS

Binding of AP2, AP3, and Tab to intact human platelets. To investigate the spatial relationship of the binding sites of AP2, AP3, and Tab to the GPIIb-IIIa complex, we examined the ability of these three monoclonal antibodies to compete with each other for binding to the platelet surface. Unlabeled antibodies were added in saturating amounts in conjunction with subsaturating levels of either the same or different radioiodinated antibody, and the effects on the counts per minute bound were examined. This experimental design allowed us to conserve the amount of radioactive sample added to each tube without compromising the necessity of saturating the platelet surface with at least one of the antibodies to determine true competition. It was determined that the dissociation constants of the three monoclonal antibodies would not be a factor in performing the competition experiments since they each have similar affinities for the platelet surface (Table 1). Figure 1 shows a representative inhibition experiment using radiolabeled AP2 and competing levels of each of the other monoclonal antibodies. As can be seen, ¹²⁵I-AP2 binding was inhibited by excess unlabeled AP2 but not at all by either Tab or AP3. Of special importance is the observation that even the combination of saturating amounts of AP3 and Tab had no effect on the binding of subsaturating amounts of AP2. Similar experiments using radiolabeled AP3 or radiolabeled Tab (not shown) showed that the binding of each radiolabeled monoclonal antibody could only be effectively blocked by its

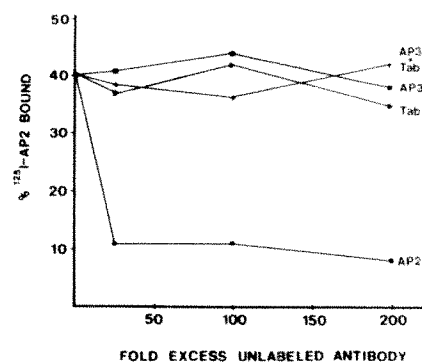


Fig 1. Simultaneous binding of AP3, AP2, and Tab to washed, intact human platelets. Radiolabeled AP2 (3.6 ng) was added to each tube in the presence or absence of a variable excess of the indicated unlabeled monoclonal antibody. The final volume was adjusted with PRP to 300 μ L to yield a platelet concentration of 2×10^7 /mL.

corresponding unlabeled antibody. In no instance was binding affected by the presence of saturating levels of the other two. Thus, these data indicate that the GPIIb-IIIa complex is able to accommodate the simultaneous binding of at least three different intact monoclonal IgG antibodies and that the epitopes recognized by AP2, AP3, and Tab are sufficiently distinct to permit cohabitation.

Effect of AP2, AP3, and Tab on ADP- or thrombin-induced platelet aggregation and secretion. We next examined the effects of AP3 and Tab, alone or in combination, on platelet aggregation. As shown in Fig 2, neither AP3 nor Tab alone inhibited ADP-induced shape change or aggregation, even at antibody doses as high as 40 μ g/mL. When the two antibodies were added together, however, platelet aggregation was completely inhibited, thereby mimicking the effect of AP2 alone. To see whether the effect of AP3 plus Tab was not merely to reduce the size of the platelet aggregate to a level that would not be recorded by the aggregometer (eg, from 10 to 4 or 5 platelets/aggregate), we verified by phase-contrast microscopy that platelets

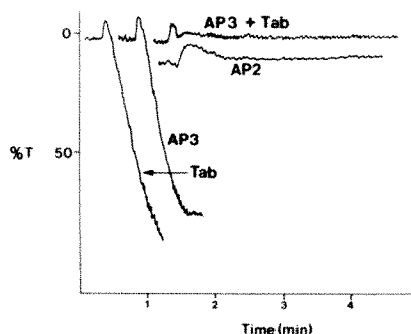


Fig 2. Effect of AP2, AP3, or Tab on ADP-induced platelet aggregation. Washed platelets were resuspended to a concentration of 1×10^8 /mL and warmed to 37 °C. Ten micrograms of the indicated monoclonal antibody was then added and allowed to incubate with stirring for five minutes. Platelet aggregation was then induced by the sequential addition of purified Peak I fibrinogen to 200 μ g/mL and ADP to a final concentration of 10 μ mol/L. The addition of fibrinogen prior to the addition of antibody did not affect the outcome of the experiment.

remained monodispersed following ADP stimulation in the presence of either AP2 or the combination of AP3 plus Tab (not shown). In both cases, shape change was readily apparent, even in the absence of platelet-platelet interaction. Concentrations of ADP as high as 30 $\mu\text{mol/L}$ were unable to overcome the inhibitory effect of AP3 and Tab. The same results were obtained using ADP/epinephrine or thrombin (0.1 to 1.0 U/mL) as agonists. When AP3 was added simultaneously with the antiplatelet monoclonal antibody AP1 (specific for GPIb), platelets were able to aggregate fully regardless of the agonist used (data not shown). Similarly, the combination of Tab and AP1 also failed to inhibit aggregation. These results indicated that inhibition was specific for the combination of AP3 plus Tab and not due to overloading of the platelet surface with immunoglobulin. Tab plus AP3 blocked platelet aggregation in a dose-dependent manner, with half-maximal inhibition occurring at 4 $\mu\text{g/mL}$ of each antibody and complete inhibition occurring at 10 to 12 $\mu\text{g/mL}$ each.

The effects of AP3 and Tab, alone or in combination, on platelet secretion were examined in a lumiaggregometer by using the luciferin/luciferase system to measure the adenosine triphosphate (ATP) released from platelet dense granules. Platelets in PRP at a final concentration of $1 \times 10^8/\text{mL}$ were preincubated with antibodies or buffer for ten minutes at 37 °C. Platelet activation was induced by the addition of 10 $\mu\text{mol/L}$ ADP, 10 $\mu\text{mol/L}$ ADP/2.2 $\mu\text{mol/L}$ epinephrine, or 0.1 units of thrombin, and the effects on both aggregometry and ATP secretion were observed simultaneously. As expected, ADP-induced ATP release was dependent upon a prior primary aggregation response; ie, AP2 alone or AP3 plus Tab blocked primary aggregation and the subsequent release of ATP. In contrast, thrombin-induced ATP release, which precedes aggregation, was unaffected by all monoclonal antibodies tested, alone and in combination, which suggests that AP2 and AP3 plus Tab block ATP release mainly by inhibiting events that lead up to secretion and do not interfere with secretion per se. The results of these studies are summarized in Table 2.

We also examined the effect of AP3 plus Tab on ADP-induced α -granule secretion as measured by β -TG release. In two experiments, AP2 alone and AP3 plus Tab each inhib-

ited β -TG release by 77% and 68%, respectively, compared with control platelets preincubated with buffer alone rather than antibodies. If PGE₁ was added prior to the addition of agonist, β -TG release was completely blocked. Similarly, in the absence of both PGE₁ and ADP, no release occurred.

Effect of AP2, AP3, and Tab on ADP-induced fibrinogen binding. Since AP3 and Tab clearly bind to different epitopes than AP2 (Fig 1) yet together inhibit aggregation to a similar extent (Fig 2), we examined the effects of the combination of AP3 plus Tab on ADP-induced fibrinogen binding. As has been reported previously, AP2 inhibits platelet aggregation and fibrinogen binding to the platelet surface,¹⁵ whereas Tab alone¹⁶ and AP3 alone¹⁹ are without effect. We confirmed these observations, and our results are shown in the inset to Fig 3. Surprisingly, the combination of AP3 plus Tab, even at concentrations as high as 20 $\mu\text{g/mL}$ each, also failed to inhibit the binding of fibrinogen to platelets of all concentrations tested. As shown in Fig 3, the binding isotherm for fibrinogen interaction with the platelet in the presence of AP3 plus Tab is nearly superimposable with the binding curve generated in the absence of antibodies. This observation has been confirmed using platelets from seven different individuals. Aggregometry was always performed in parallel with fibrinogen-binding experiments, using identical reagents and antibody preparations to eliminate variations that might bias their comparison, and aggregation was always completely inhibited under conditions where fibrinogen binding was 100%.

To test whether fibrinogen might be bound abnormally in the presence of these two antibodies, quantitative analyses of several additional binding experiments were performed. Both the number of fibrinogen molecules bound at saturation and the affinity of fibrinogen for its platelet receptor were determined in the presence or absence of 20 $\mu\text{g/mL}$ each of AP3 and Tab. Scatchard plot analysis of the binding data yielded linear plots, with no evidence of cooperativity or multiple classes of receptor sites. The mean K_d for fibrinogen binding was calculated to be $0.52 \pm 0.14 \mu\text{mol/L}$ (mean $\pm$ SEM of three determinations using two different donors) in the absence of antibody and $0.32 \pm 0.06 \mu\text{mol/L}$ in the presence of saturating amounts of Tab and AP3. Similarly, no significant difference was observed in the number of

Table 2. Summary of the Effects of AP2, AP3, and Tab on Selected Platelet Functions

Agonist	Antibodies	Fibrinogen Binding	Percent Aggregation	Percent ATP Release	Shape Change
ADP	None	+	100	100	+
	AP3	+	100	100	+
	Tab	+	100	100	+
	AP3 + Tab	+	0	0-5	+
	AP2	—	0-10	0	+
	Fabs $\pm$ G α M IgG	ND	100	100	+
Thrombin	None	+	100	100	+
	AP3	+	50-100	100	+
	Tab	+	100	100	+
	AP3 + Tab	—	0-5	100	+
	AP2	—	0	0	+
	Fabs $\pm$ G α M IgG	ND	100	100	+

All results are discussed in detail throughout the text.

Abbreviations: +, a normal response; ND, not done; G α M IgG, goat antimouse IgG.

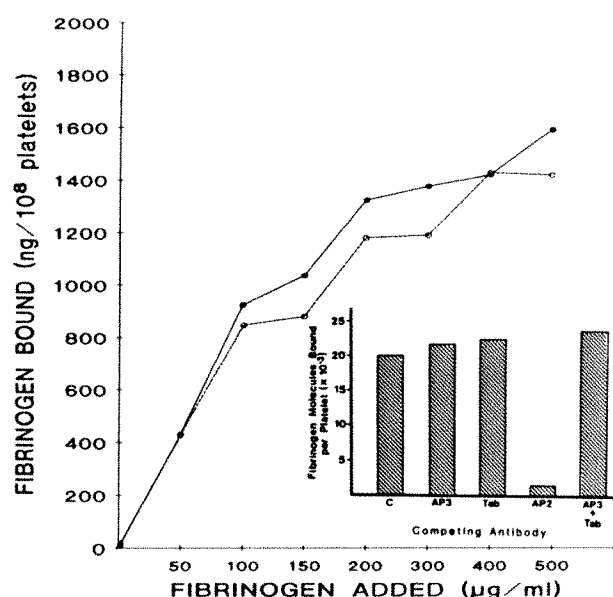


Fig 3. ADP-induced fibrinogen binding to intact platelets in the presence or absence of AP3 plus Tab. ^{125}I -fibrinogen was added in increasing concentrations to washed platelets ($1 \times 10^8/\text{mL}$) and allowed to incubate at 25°C for five minutes in the presence (○—○) or absence (●—●) of $20 \mu\text{g}/\text{mL}$ each of AP3 and Tab. Binding was induced by the addition of ADP to $10 \mu\text{mol}/\text{L}$. Nonspecific binding was subtracted as described in Materials and Methods. Values shown represent the specific fibrinogen binding from one typical analysis. (Inset) Histogram depicting the individual effects of AP2, AP3, Tab, and AP3 plus Tab on ADP-induced ^{125}I -fibrinogen binding. Platelets were at a $1 \times 10^8/\text{mL}$ and fibrinogen at a $200 \mu\text{g}/\text{mL}$ final concentration. All antibodies were at $10 \mu\text{g}/\text{mL}$ each. Binding was induced by the addition of ADP to $10 \mu\text{mol}/\text{L}$. c, control samples with no antibody. Note that AP3 plus Tab completely block platelet aggregation (Fig 2) without inhibiting fibrinogen binding.

fibrinogen molecules bound per platelet at saturation in the presence or absence of the two antibodies (Table 3). Significant differences in the number of fibrinogen binding sites per platelet between individual donors was observed (donor A, approximately 50,000 sites; donor B, approximately 20,000 sites), which confirms previous reports.^{14,24,25}

Both Fig 3 and Table 3 contain data derived from quantitative binding analyses performed under nonstirring conditions at 25°C . Two additional fibrinogen binding experiments were performed at 37°C under nonstirring conditions

Table 3. Quantitative Effect of AP3 and Tab on the Interaction of Fibrinogen With Platelets

Donor	Antibodies	Dissociation Constant ($\mu\text{mol}/\text{L}$)	Molecules Bound/Platelet
A	None	0.50 ± 0.14	$54,300 \pm 7,600$
A	AP3 + Tab	0.32 ± 0.06	$42,400 \pm 3,700$
B	None	0.54 ± 0.14	$19,800 \pm 2,650$
B	AP3 + Tab	0.33 ± 0.06	$17,400 \pm 1,300$

^{125}I -fibrinogen was added in increasing amounts to platelets ($1 \times 10^8/\text{mL}$) until saturation was reached. AP3 and Tab, when present, were at $20 \mu\text{g}/\text{mL}$ final concentration each. Binding data were analyzed using the weighted, nonlinear curve fitting program LIGAND. Values reported are the mean of three determinations $\pm$ SE using platelets from two different donors, A and B.

in test tubes. The results obtained at 37°C (not shown) were nearly identical to those shown in Fig 3 except that the total number of fibrinogen molecules bound to the platelet surface at saturation was in each case reduced by approximately 50%. This effect of temperature on fibrinogen binding has been previously documented in detail by Marguerie and Plow.²⁶ To ensure that fibrinogen binding to platelets in the presence of AP3 plus Tab also occurred at 37°C under stirring conditions, we performed three additional experiments in which we measured fibrinogen binding directly in an aggregometer with stirring at 1,000 rpm at 37°C . The results obtained in the aggregometer are comparable to those obtained in a test tube, though we did see a higher degree of nonspecific binding and trapping within platelet aggregates as was expected. As shown in Table 4, AP3 plus Tab did not inhibit the number of ^{125}I -fibrinogen molecules that become associated with platelets, whereas AP2 or EDTA both significantly reduced fibrinogen binding. Aggregation was completely blocked by EDTA, AP2, or AP3 plus Tab, as before. Thus, inhibition of aggregation by AP3 plus Tab seems to occur in the presence of apparently normal fibrinogen binding under all conditions tested (ie, 25°C and 37°C , stirring or nonstirring).

Effects of AP3 and Tab on thrombin-induced fibrinogen binding. To determine whether the effects of AP3 plus Tab on fibrinogen binding were limited to ADP-induced activation, we repeated the aforementioned studies using thrombin as the agonist. As discussed earlier, thrombin-induced platelet aggregation was completely inhibited by AP2 or AP3 plus Tab but not at all by AP3 or Tab alone, analogous to our observations using ADP as the agonist. Neither AP3 alone nor Tab alone inhibited the binding of fibrinogen to thrombin-stimulated platelets, also corresponding to our observations using ADP as the agonist. When platelets were preincubated with AP3 plus Tab, thrombin-induced fibrinogen binding was completely blocked, thereby mimicking the action of AP2 alone (Fig 4). When platelets were activated with thrombin/hirudin prior to rather than following incubation with AP3, Tab, and fibrinogen, fibrinogen binding was also blocked, which demonstrated that these antibodies block thrombin- but not ADP-induced fibrinogen binding when

Table 4. Semiquantitative Measurement of Fibrinogen-Platelet Association at 37°C Under Stirring Conditions

Antibody	Aggregation	Bound cpm	μg Fibrinogen Bound per 10^8 Platelets
None	+	8,000	1.3
$10 \text{ mmol}/\text{L}$ EDTA	—	5,400	0.89
AP2	—	5,424	0.89
AP3 + Tab	—	10,150	1.6

Washed platelets at $1 \times 10^8/\text{mL}$ in a volume of $400 \mu\text{L}$ were preincubated in an aggregometer under stirring conditions with $200 \mu\text{g}/\text{mL}$ radiolabeled fibrinogen and $20 \mu\text{g}/\text{mL}$ of each indicated monoclonal antibody for ten minutes at 37°C . Platelet activation was initiated by the addition of ADP up to $20 \mu\text{mol}/\text{L}$. After five minutes, $75 \mu\text{L}$ was withdrawn and centrifuged through $500 \mu\text{L}$ of 15% sucrose to separate bound from free ligand. Following aspiration of the supernatant, the radioactivity associated with the platelet pellet was determined in a gamma counter. Specific activity of the labeled fibrinogen was $81,000 \text{ cpm}/\mu\text{g}$.

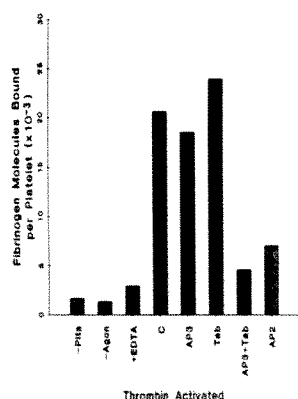


Fig 4. Effect of AP3 and Tab on fibrinogen binding to thrombin-activated platelets. Binding conditions were identical to those described for the inset to Fig 3 except that 0.1 U/mL of α -thrombin was used as the agonist and all incubations were performed at 37 °C rather than 25 °C. -Plts, no platelets added, representing nonspecific binding to the incubation tube; -Agon, no ADP or thrombin added; +EDTA, platelets incubated with agonist but in the presence of 5 mmol/L EDTA; c, control tube containing platelets and agonist. Antibodies added where indicated were at final concentrations of 10 μ g/mL each. Note that AP3 plus Tab block thrombin- but not ADP-induced fibrinogen binding (ie, compare Fig 3 inset with this Fig). Platelet aggregation is inhibited by using either agonist.

bound to either the activated or inactivated platelet surface. These data are summarized in Fig 4, and the effects of these antibodies, alone and in combination, on selected platelet functions are summarized in Table 2.

Effect of AP3 and Tab Fab fragments on platelet function. The monoclonal antibodies used in all of the aforementioned experiments were in the form of intact IgG, which by analogy with other antireceptor antibodies have the ability to interact with two identical epitopes, thus raising the possibility of cross-linking nearby membrane receptors. To determine whether lateral cross-linking of adjoining GPIIb-IIIa complexes might play a role in the synergistic action of AP3 and Tab on inhibition of platelet aggregation, monovalent Fab fragments were prepared and used in studies comparable to those reported above. Fab fragments of both AP3 and Tab were found to have similar binding affinities for the platelet surface as their corresponding intact IgGs, as judged by their effectiveness in competing for radiolabeled intact IgGs. Like their intact counterparts, Fab fragments of AP3 alone or Tab alone had no effect on ADP- or thrombin-induced platelet aggregation or secretion. Unlike intact IgG, however, the combination of Fab fragments of both AP3 and Tab at concentrations of up to 50 μ g/mL each also was without effect on platelet function. Interestingly, the combinations of intact AP3 IgG together with Tab Fab fragments or intact Tab IgG together with AP3 Fab fragments also were unable to inhibit platelet aggregation or secretion induced by either ADP or thrombin. Thus, it appears that both antibodies must be divalent for them to exert their synergistic inhibitory effect on platelet aggregation and secretion. To test whether the use of Fab fragments was not merely removing a steric block that intact IgGs might place on fibrinogen binding, thereby allowing platelets to aggre-

gate, platelets were additionally incubated with 50 μ g/mL of affinity purified, fluorescein isothiocyanate-conjugated, goat antimouse IgG subsequent to saturating the platelet surface with Fab fragments of AP3 and Tab. Binding of the second antibody was verified by immunofluorescent microscopy and did not occur without prior incubation of platelets with murine antibodies. Under these conditions, ie, with both Fab fragments and intact IgGs on and near the platelet fibrinogen receptor, platelet aggregation and secretion still proceeded normally. Binding of goat antimouse IgG to intact AP3 and Tab on the platelet surface did not affect their ability to block platelet aggregation and secretion, thus ruling out any effect on platelet aggregation by the second antibody alone. These studies are summarized in Table 2.

DISCUSSION

There is now compelling evidence that membrane GPIIb and IIIa are the major components of the human platelet fibrinogen receptor. First, platelets from patients with type I Glanzmann's thrombasthenia, which contain less than 5% of the normal levels of GPIIb-IIIa, fail to bind detectable levels of fibrinogen following stimulation with ADP.^{5,13,14,25} Second, monoclonal antibodies raised against the GPIIb-IIIa complex mimic the thrombasthenic condition by inhibiting both fibrinogen binding and platelet aggregation.¹⁵⁻¹⁸ Third, UV irradiation of photoaffinity-labeled fibrinogen resulted in its incorporation into GPIIb.²⁷ Fourth, Nachman and Leung have demonstrated complex formation of purified GPIIb-IIIa with fibrinogen attached to plastic microtiter wells.²⁸ Gogstad et al²⁹ using crossed immunoelectrophoresis have shown that radiolabeled fibrinogen binds to immunoprecipitin arcs containing the intact GPIIb-IIIa complex but not to precipitin arcs given by the dissociated glycoproteins. Finally, purified GPIIb-IIIa complexes incorporated into phospholipid vesicles retain the ability to bind fibrinogen in a dose-dependent, saturable manner.^{30,31}

In this report, we have used three well-characterized monoclonal antibodies directed against the components of the platelet fibrinogen receptor to further investigate the molecular requirements for fibrinogen binding and platelet-platelet cohesion. Using a series of complimentary competitive inhibition experiments, we showed that AP2, AP3, and Tab did not compete with each other in binding to the GPIIb-IIIa complex of intact platelets. Moreover, the combination of AP3 and Tab did not inhibit the binding of AP2 to its GPIIb-IIIa complex-specific determinant. Taken together, it appears that three different monoclonal antibodies (150,000 daltons each) can occupy a single platelet fibrinogen receptor (molecular weight about 230,000) simultaneously.

Both ADP-induced platelet aggregation and secretion were unaffected by saturating levels of either AP3 or Tab. When these two antibodies were added together, however, both of these processes were completely inhibited, mimicking the effects of AP2 alone. In light of the fact that AP2 alone or AP3 plus Tab inhibited ADP-induced platelet aggregation, it was not surprising that secretion was also suppressed because ADP-induced release is known to occur subsequent to and be dependent upon the occurrence of primary aggre-

gation.^{24,32-34} Since ADP-induced fibrinogen binding and primary aggregation are also normal in aspirin-treated platelets,³⁵ which also are incapable of secretion, it is obvious that the failure of platelets to aggregate in the presence of AP3 plus Tab is due to something other than inhibition of the release reaction. Thrombin-activated platelets also did not aggregate in the presence of AP3 plus Tab or AP2 alone, though the release reaction, which unlike ADP activation occurs prior to aggregation, was unaffected. Thus, these data provide further evidence that induction of secretion alone is insufficient stimulus to initiate platelet aggregation.

It is noteworthy that AP3 and Tab exert their synergistic inhibitory effect on both ADP- and thrombin-induced platelet aggregation through two apparently distinct mechanisms. When ADP is used to activate platelets, fibrinogen binding is both quantitatively and qualitatively normal insofar as we have been able to measure (Table 3 and Fig 3), yet aggregation and release are totally blocked (Fig 2, Table 2). Thrombin-activated platelets, however, neither bind fibrinogen (Fig 4) nor aggregate in the presence of AP3 plus Tab, yet the release reaction is normal. That thrombin initiates surface membrane changes at or near the platelet fibrinogen receptor distinct from that generated by ADP activation is further supported by previous studies showing that (1) thrombin, but not ADP, is able to expose binding sites for fibronectin on the platelet IIb-IIIa complex³⁶; (2) thrombin, but not ADP, induces thrombospondin binding to platelets³⁷; and (3) ADP- but not thrombin-induced Ca^{+2} influx is inhibited by two different GPIIb-IIIa complex-specific monoclonal antibodies.³⁸ Although none of these experiments, including the ones presented in this manuscript, are completely understood at a molecular level, taken together they emphasize the complexity and multiple modes by which the platelet surface can become activated.

The observation that ADP-induced platelet aggregation is completely blocked in the presence of apparently normal fibrinogen binding deserves further comment. There are several plausible explanations why platelet cohesion might be inhibited although binding of fibrinogen to the platelet surface has occurred. We cannot totally exclude the possibility that fibrinogen binding is somehow qualitatively altered when Tab and AP3 occupy sites on GPIIb and GPIIIa, though several lines of evidence argue against this. If the binding of fibrinogen to the platelet surface was anomalous in the presence of AP3 plus Tab or if fibrinogen was interacting with either of the two antibodies, a change in either the number of fibrinogen molecules bound, the affinity of fibrinogen for the platelet surface, or both would be detected. Our data (Fig 3 and Table 3) demonstrate that neither of these two binding parameters were altered in the presence of one or both of these two monoclonal antibodies, indicating that other modes of inhibition must be considered.

Since Fab fragments of AP3 or Tab are unable to inhibit platelet aggregation even when only one of the two antibodies used is a Fab, these studies suggest that AP3 and Tab exert their synergistic inhibitory effect on ADP-induced platelet aggregation and secretion by a divalent antibody-dependent mechanism rather than by sterically modifying the way in which fibrinogen binds to the platelet surface. The failure of an intact second antibody bound to multiple sites of surface-bound Fab fragments of AP3 and Tab to reconstitute the inhibition of ADP-induced platelet aggregation further argues against simple steric hindrance as the mechanism by which AP3 Tab inhibits platelet aggregation.

Inasmuch as ADP-induced fibrinogen binding appears to be unaffected by AP3 plus Tab, it is likely that the lack of aggregation in the presence of these two antibodies represents the inhibition of a physiologically important event that occurs subsequent to fibrinogen binding to the platelet surface. In this regard, monoclonal antibodies directed against either thrombospondin³⁹ or fibronectin⁴⁰ have also been reported to inhibit platelet aggregation by unknown mechanisms. It is possible that fibrinogen-fibrinogen associations between adjacent platelets may play a role in platelet cohesion and antithrombospondin, antifibronectin, and AP3 plus Tab all block platelet aggregation by decreasing the affinity of fibrinogen-coated platelets for each other. On the other hand, by analogy with other cell surface receptors, eg, those for insulin or IgE, it is reasonable to speculate that reorganization and/or clustering of cell surface molecules might play a role in platelet activation. Employing diverse methodologies including immunoelectron microscopy,⁴¹⁻⁴⁴ chemical cross-linking,⁴⁵ and kinetic analyses of fibrinogen binding,³⁵ several investigators have found evidence for interaction of fibrinogen receptors following platelet activation. The combination of AP3 and Tab may somehow interfere with activation-induced association of fibrinogen receptors with each other or the underlying cytoskeleton. It is noteworthy that Estry and colleagues⁴⁶ have recently reported that fibrinogen binds normally to platelets of dogs with congenital thrombopathia, a hereditary disorder of platelet function characterized by a thrombasthenialike defect in aggregation but quantitatively normal levels of GPIIb-IIIa.^{47,48} The intriguing possibility exists that AP3 plus Tab are introducing a defect similar to that found in canine thrombopathia. Using immunoelectron microscopy in conjunction with other methods, we plan to address questions concerning the interaction and possible reorganization of fibrinogen receptors following platelet activation.

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Characterization of Monoclonal IgG Cryoglobulins: Fine-Structural and Morphological Analysis

By D.N. Podell, C.H. Packman, J. Maniloff, and G.N. Abraham

The morphology of the amorphous, gelatinous, and crystalline varieties of monoclonal IgG cryoglobulins was analyzed by light and transmission and scanning electron microscopy. Each cryoglobulin had a characteristic fine structure that correlated with its gross morphology. Transmission electron microscopy showed that the amorphous precipitates were random and disorganized molecular clumps. In contrast, cryogels were thin-walled, well-organized, and hydrated strawlike clusters, whereas cryocrystals formed tightly compacted, highly structured molecular clusters.

MONOCLONAL HUMAN IgG cryoglobulins without defined antibody activity have unique immunologic and biochemical characteristics.¹⁻³ Their primary,⁴ secondary, and tertiary⁵⁻⁷ structural properties cause self-association and precipitation from chilled solutions by diverse biophysical processes.⁵⁻¹² The cryoglobulinemic syndrome resulting from these molecular interactions is caused by cryoglobulin polymerization or precipitation *in vivo*, which produces signs of complete or partial small-vessel occlusion with or without accompanying hyperviscosity. Due to differences in their structural and biophysical properties, IgG cryoglobulins may assume amorphous, gelatinous, and/or crystalline morphological forms *in vitro*, either in the patient's serum or in solution after purification.¹³

The severity of the cryoglobulin-related clinical disease is related to the morphology of the IgG cryoprecipitate and the nature of the *in vivo* aggregate or molecular complex that forms. Clinically, the vascular occlusion causes skin lesions that range from superficial purpura to deep gangrenous ulcers in dependent areas. In general, the most severe symptoms are associated with crystalline cryoprecipitates⁴ and the mildest with gelatinous cryoprecipitates.

Although the processes of cryogel and the cryocrystal self-association and subsequent precipitation differ,^{5-7,10} differences in the structure of the various types of cryoprecipitates have not been previously described. In this report, monoclonal IgG cryoglobulins with defined biophysical properties were purified from patient sera and studied by light and scanning and transmission electron microscopy to determine their ultrastructural properties. These findings may clarify, in part, how these proteins produce their diverse clinical symptomatology.

MATERIALS AND METHODS

DUB (IgG₁K), WEB (IgG₁), and LON (IgG₁) were purified from each patient's serum as previously described.^{3,13} Each formed a visibly different cryoprecipitate after incubation of the serum at 4 °C for seven days. IgGs DUB and WEB both produced flocculant white precipitates. After gentle manual inversion, the WEB cryoprecipitate appeared finely dispersed, and the solution showed a silky sheen characteristic of suspensions of microcrystals. The cryoprecipitate formed by IgG DUB was more flocculant, appeared in visible clumps (0.1 to 0.25 mm in size), and did not show the sheen noted for WEB. IgG LON formed a firm, opaque, cryogel that occupied the entire serum volume. The tube in which the LON cryogel formed was able to be completely inverted with minimal loss of serum.

Crystals that formed in blood produced rouleaux, and analysis by scanning electron microscopy indicated that the crystals could form thick-walled, branching, macromolecular nets that could physically trap cells. The morphological properties provided visual impressions by which cryoglobulins could cause clinical disease secondary to vascular occlusion produced by self-associated IgG cryoglobulin molecules.

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The amorphous and crystalline cryoglobulins and a portion of cryogel LON were gently dispersed in ice-chilled phosphate-buffered saline (PBS) (0.01 mol/L phosphate buffer, 0.154 mol/L NaCl, pH 7.4) and collected by centrifugation at 200 rpm for 20 minutes at 4 °C. Each was then dissolved in PBS at 37 °C and reprecipitated by 24 to 48 hours of incubation at 4 °C. This process was repeated four times to rid the cryoglobulins of trace contaminants of albumin. IgG LON was additionally dialyzed against 0.015 mol/L phosphate, pH 7.4, at 37 °C and passed through a diethyl aminoethyl column (equilibrated with the same buffer) at 37 °C. All three proteins were judged immunologically pure by immunoelectrophoresis with antisera specific for the human heavy-chain classes and light-chain types and produced isoelectric focusing profiles with markedly restricted heterogeneity, typical of purified myeloma proteins.³ In addition, isolated heavy and light chains yielded single aminoterminal primary amino acid sequences upon automated Edman degradation.³

For electron microscopy, purified cryoglobulins were allowed to reform at 4 °C in PBS, gently resuspended and added dropwise to ice-chilled 0.1% glutaraldehyde in 0.15 mol/L phosphate buffer, pH 7.4. The cryoglobulins were allowed to settle for 20 minutes and then collected by centrifugation at 200 rpm at 4 °C. Fixed cryoproteins were washed several times after resuspension in chilled 0.15 mol/L phosphate buffer (pH 7.4), postfixed in osmium tetroxide, alcohol dehydrated, and embedded in epoxy resin.

For transmission electron microscopy, 50 nm sections were stained with uranyl acetate prior to examination. The technique is similar to that described by Stoeber et al¹⁴ except that a lower concentration of glutaraldehyde was used.

Image analysis of micrographs was done using a 6-m optical bench with a 5-mW He-Ne laser as described previously.¹⁵

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Dedicated to the memory of Dr Robert Weed.

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RESULTS

Light microscopy studies. Citrated blood samples from patients DUB, WEB, and LON were incubated at 4 °C for one hour. The tubes were mixed by gentle inversion to resuspend settled cells, and one drop from each sample was placed on a microscope slide. A cell monolayer was produced by spreading, and the cells were then stained with Wright's stain and examined. Samples from patients DUB and LON were unremarkable. However, that of WEB contained numerous rouleaux. As shown in Fig 1A, the erythrocyte clusters have crystals at their core. Figure 1B shows fusiform crystals of WEB, which formed in serum after ten minutes incubation at 4 °C and are morphologically similar to those noted in the centers of the rouleaux.

The following experiments demonstrated that rouleau formation was not due to agglutination of erythrocytes secondary to an antierythrocyte specificity for IgG WEB: (1) Noncryoprecipitating F(ab')₂ fragments prepared by pepsin digestion of IgG WEB did not hemagglutinate, whereas anti-Rh antibody did hemagglutinate washed WEB red cells in a microtiter hemagglutination assay.¹⁶ (2) Incubation of washed WEB red cells with ¹²⁵I-labeled F(ab')₂ of IgG WEB showed no greater uptake of label than did ¹²⁵I-labeled F(ab')₂ prepared from pooled normal human IgG that had been absorbed with both pooled human erythrocytes prior to pepsin digestion and IgG WEB bound to bromoacetyl cellulose.¹⁷ The background binding amounted to less than 1% of the total radioactivity added to the assay mixture.

Transmission electron microscopy. The cryoprecipitates were examined by transmission electron microscopy. Figure 2A shows the amorphous nature of IgG DUB.

Although the DUB cryoprecipitate has no obvious gross structure or molecular regularity, close examination reveals rare structures with the dimensions and shape of individual IgG molecules. Prolonged cultivation of the cryoprecipitate for up to 1 month in solutions of increasing salt concentrations (0.01 to 0.5 mol/L NaCl) did not change the amorphous nature of IgG DUB.

The cryogel IgG LON is shown in Fig 2B, C, and D. After purification, the LON cryoprecipitate formed narrow fibrils. Longitudinal cross sections of these fibrils (Fig 2C) show thin-walled, hollow tubules. In all sections studied, the tubules are organized into bundles that were seen in both horizontal (Fig 2B) and oblique (Fig 2D) cross-sectional cuts. The lack of density suggests a diffuse molecular structure containing a large amount of trapped water. Using polarized light, unfixed preparations of IgG LON were not refractive, in agreement with the lack of molecular order. No definite molecular organization or structure was noted in high-magnification photomicrographs.

In contrast, washed and purified WEB cryoprecipitates spontaneously formed microcrystals that were birefringent with plane polarized light. Bright-field light microscopy showed these microcrystals to be elongated and strawlike, either single or in bundles, from 20 to 400 or 500 μm in length.¹³ High-power electron micrographs of perpendicular, parallel, or oblique cuts to the long axis of the WEB crystals showed variations of the dense herringboned, cross-hatched pattern noted in the longitudinal sections shown in Fig 3. This pattern results from the self-association of IgG WEB molecules into highly organized and tightly packed crystals. Close examination shows individual IgG molecules with a

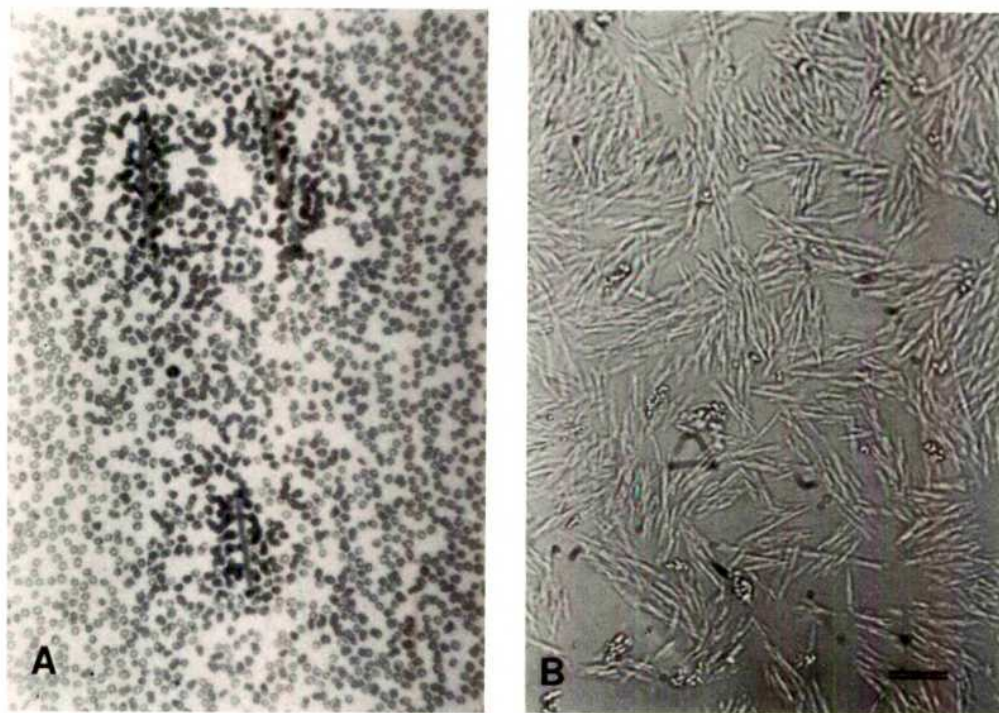


Fig 1. (A) Light micrograph of IgG WEB cryocrystals formed by the incubation of citrated blood at 4 °C. Elongated crystals are surrounded by erythrocyte clumps. (B) Light micrograph of IgG WEB cryocrystals formed by the incubation of plasma at 4 °C for 24 hours. The feathery crystals range from 50 to 250 μm in size. Bar A, B, 67 μm.

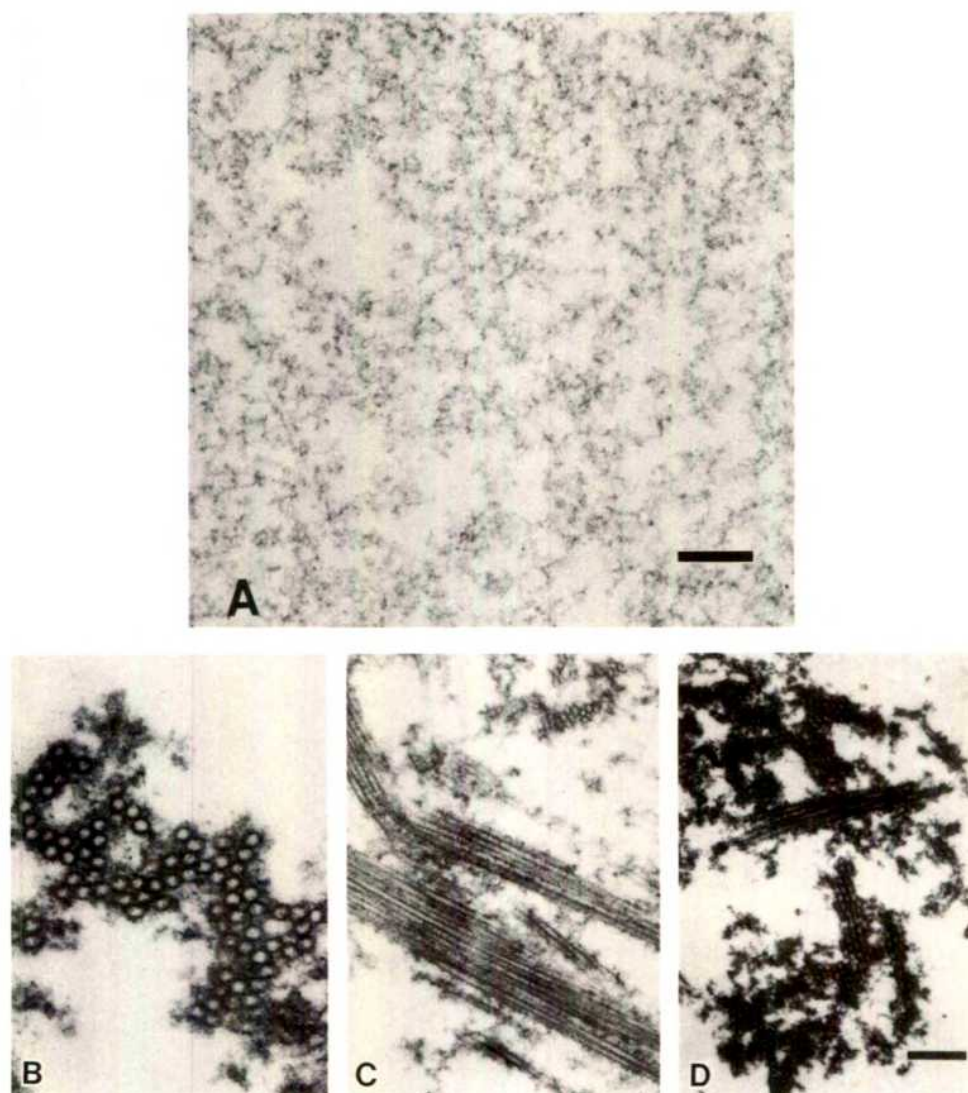


Fig 2. (A) Transmission electron micrograph of amorphous IgG cryoglobulin DUB. No defined pattern or molecular array is noted. Bar, 0.5 μm . (B, C, and D) Transmission electron micrographs of cryogel IgG LON that demonstrate its fibrillar and tubular properties. B, C, and D represent perpendicular, longitudinal, and oblique sections of the fibrils. Bar B, 0.25 μm ; C, 0.55 μm ; D, 0.625 μm .

typical "Y" configuration. Optical diffraction analyses of 15,000 $\times$ electron micrograph negatives showed the units (IgG molecules) in the microcrystals to be in a helical arrangement with a periodicity of six to eight molecules and longitudinal and transverse dimensions of 140 $\text{\AA}$ and 100 $\text{\AA}$, respectively. The latter measurement represents the computed distance between the amino terminal ends of the two limbs of the f(ab) regions of an IgG molecule.

Scanning electron microscopy. Scanning electron microscopy of crystals of IgG WEB showed dense walls of the WEB crystals whose tips are sometimes irregular, fractured, and jagged (Fig 4D) and other times blunt and thick with rounded and curled over edges (Fig 4A). This side-to-side association produces the sheets of tubules shown in Fig 4A. The ability of the WEB crystals to branch and form smaller projections that stem from larger crystal trunks is seen in Fig 4B. The branching and intercollation can result in the netlike tangled array of crystals shown in Fig 4C.

To determine whether WEB crystals had the ability to trap blood cells, 10^7 washed WEB peripheral red and white blood cells in PBS, pH 7.4, were added to 1 mL of a warmed (37 $^{\circ}\text{C}$) solution of purified WEB cryocrystals (concentration, 1.5 mg/mL). The mixture was incubated at 4 $^{\circ}\text{C}$ for 24 hours. The cryocrystals and cells then were allowed to settle and the cell-crystal sediment prepared for electron microscopy. Figure 5 shows a photomicrograph of a single red cell enveloped by the WEB cryocrystal filaments. The photomicrograph demonstrates that cryocrystals sometimes can form around, physically trap, and immobilize cells in the interstices of the cryoprotein net.

DISCUSSION

The present studies demonstrate that the three morphological types of monoclonal IgG cryoglobulins form precipitates that have different fine ultrastructural characteristics. As shown by electron microscopy, cryoglobulins may self-



Fig 3. Transmission electron micrograph of a longitudinal section of cryocrystal IgG WEB. The dense herringbone pattern is produced by highly organized and tightly packed IgG molecules. Bar, 0.13 μm .

associate in many ways: (1) as amorphous cryoprecipitates without any discernible molecular organization; (2) as cryogels comprised of loosely organized tubular structures that appear highly hydrated; and (3) as cryocrystals that are dense, compact, highly organized structures with a periodic molecular array.

These studies also reveal another characteristic of IgG cryogel and cryocrystal globulins, namely, that they may amplify their size by organization of the basic tubular gel or crystalline units into large bundles. The size and complexity of the cryoprecipitate is enhanced by the ability of both the cryogel and cryocrystals to bend and also to form structural branch points from the larger cryoprotein trunks of the precipitate. For the cryocrystal this process produces a complex and dense cryoprotein network that may be associated with cellular aggregation. This is visually noted by the

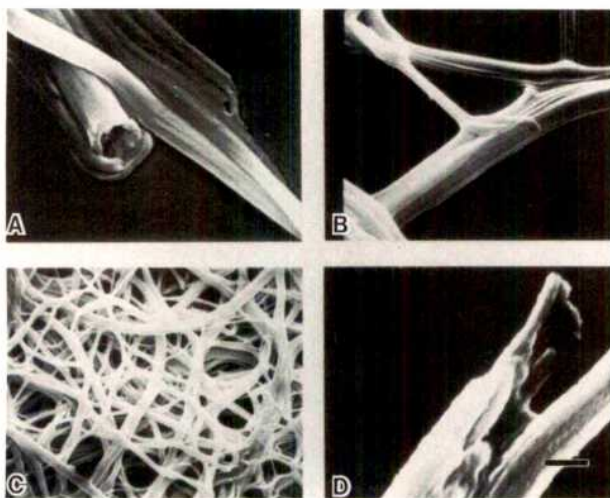


Fig 4. Scanning electron micrographs of IgG WEB crystals. The micrographs demonstrate the ability of the crystals to branch and form nets and their thick tubular structure. Bar A, 1 μm ; B, 1.5 μm ; C, 4 μm ; D, 0.5 μm .

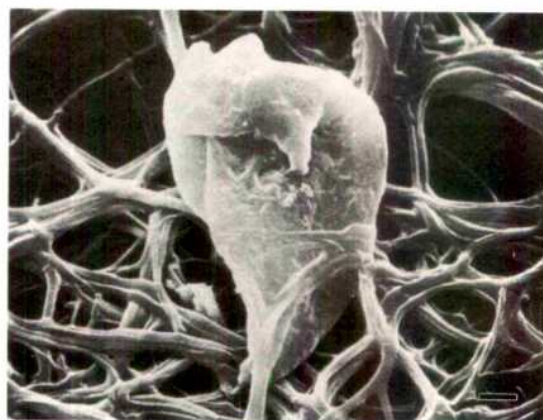


Fig 5. Scanning electron micrograph of an erythrocyte entangled in the IgG WEB cryocrystalline net. Bar, 0.8 μm .

presence of the erythrocyte rouleaux in peripheral blood that contain a single cryocrystal at their central core. The cryoglobulin net may also physically trap cells. The scanning electron micrographs of IgG WEB show that purified WEB crystals can form tentaclelike, branched crystalline projections that may, upon crystallization, envelop and ensnare blood cells.

The clinical symptoms that accompany primary idiopathic IgG cryoglobulinemia are of variable severity. The symptoms are most likely due to the presence of self-associated IgG aggregates in the peripheral vasculature.^{1,4,18} In its most severe form, a necrotizing vasculitis manifested clinically by generalized skin ulcers is noted, which may become gangrenous. This is secondary to the occlusion of small blood vessels with cryoprecipitate and accompanied by a marked perivascular and intramural arteriolar inflammatory reaction.¹³ This situation should be contrasted with tissue or organ fixation and the renal and skin lesions that are associated with the more common mixed IgM-IgG cryoglobulins. These are true antigen-antibody complexes¹⁹ and in most instances avidly fix complement.¹⁸

The morphologies for IgG WEB and IgG LON correlate with their differing biophysical properties and their contrasting mechanisms for self-association. IgG LON forms thin-walled tubules after a four- to seven-day period of cryogelation. Biophysical studies^{6,7} demonstrate that these axially asymmetric IgGs form large polymers through weak non-ionic and hydrophobic self-associative interactions. Cryocrystals such as WEB aggregate with rapid concentration-dependent kinetics^{5,10} after the formation of a nucleation core that produces a dense molecular pack. Although primary amino acid sequence analyses demonstrate diverse primary structural mutations in both heavy and light chains of these proteins,⁴ their relationship to the cryoprecipitation process has not as yet been determined.

In sum, these morphological analyses provide a visual indication of how IgG cryoglobulin molecular self-associations may produce the small-vessel vascular insufficiency and blockade characteristic of idiopathic monoclonal IgG cryoglobulinemia. In addition, the analyses show the close correlation between fine structural morphology and gross characteristics of the three cryoprotein types.

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Control of Hemopoiesis by a Bone Marrow Stromal Cell Clone: Lipopolysaccharide- and Interleukin-1-Inducible Production of Colony-Stimulating Factors

By D. Rennick, G. Yang, L. Gemmell, and F. Lee

A stromal cell line, GY30, was cloned from mouse bone marrow adherent cell layers. In culture, GY30 cells sustain the production of granulocyte-macrophage progenitor cells (GM-CFU) but fail to support the survival of pluripotential stem cells (CFU-S). GY30 cells secrete two growth factor activities distinct from interleukin-3 (IL-3), IL-2, and macrophage colony-stimulating factor (M-CSF) but functionally similar to GM-CSF and G-CSF. The production of both CSFs

is increased 70- to 200-fold by treating GY30 cells with lipopolysaccharide or IL-1. RNA blot analysis reveals the presence of GM-CSF and G-CSF transcripts and demonstrates that IL-1 regulates the production of both factors at the mRNA level. Further, these studies show that the GM-CSF secreted by GY30 cells is structurally similar to the GM-CSF produced by activated T cells.

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IT HAS BEEN RECOGNIZED for some time that bone marrow is the major hemopoietic tissue in the adult animal. This tissue is the main source of multipotential stem cells as well as mature myeloid cells that are needed daily to replace short-lived circulating blood cells.¹ The development of clonal semisolid culture assays² has provided valuable information about the growth and differentiation of hemopoietic progenitor cells in response to colony-stimulating factors (CSFs) produced by various tissues and cell lines.³ These studies have led to the identification of several types of CSFs and the purification of four mouse factors termed interleukin-3 (IL-3) or multi-CSF,^{4,5} granulocyte-CSF (G-CSF),⁶ macrophage-CSF (M-CSF),⁷⁻⁹ and granulocyte-macrophage-CSF (GM-CSF).¹⁰ However, until recently, little was known about the specific mechanisms regulating hemopoiesis within the bone marrow. Following the development of techniques for growing bone marrow cells in liquid culture,¹¹ a series of studies showed that long-term maintenance of stem cell growth and differentiation in vitro is dependent on bone marrow-derived adherent cells.^{12,13}

The adherent cell layers consist of a heterogeneous mixture of macrophages, endothelial cells, fibroblasts, and adipocytes.¹⁴⁻¹⁷ These various cell types are collectively referred to as stromal cells. Initially, there was difficulty in demonstrating the presence of soluble CSFs in these long-term bone marrow culture systems, which led to the suggestion that stromal cells may regulate hemopoiesis primarily through cell-cell interactions.¹¹ Later it was shown that low levels of CSFs are produced by stromal cell layers¹⁸⁻²³ and that they could account in part for the growth and differentiation of granulocyte and macrophage progenitor cells in Dexter-type

cultures. However, the various types of CSF produced by stromal cells and their relationship to CSFs produced by other tissues have not been clearly established. Some cells within the stromal layer also modify the response of hemopoietic stem cells by producing colony-enhancing factors²⁴ or inhibitors of differentiation-inducing factors.²⁵⁻²⁹ Thus, adherent layers can provide in vitro both stimulatory and inhibitory signals that maintain the development of pluripotential stem cells while sustaining steady-state production of differentiated progeny in a manner similar to intact bone marrow.

Recent studies have used clonal stromal cell lines to identify specific mechanisms by which stromal cells regulate stem cell growth and differentiation. Li and Johnson³⁰ have described an adherent cell clone that supports colony formation by multipotential progenitor cells. The colony-stimulating activity associated with the stromal cell line is apparently not CSF-1 (M-CSF), GM-CSF, or IL-3 (multi-CSF). It has also been reported that a preadipocyte cell line in direct contact with hemopoietic cells sustains the proliferation of hemopoietic stem cells (CFU-S).³¹ Other cloned stromal cell lines have been shown to support colony formation by hemopoietic cells specifically committed to granulocyte-macrophage lineages,³²⁻³⁷ whereas another supports erythrocyte development as well.³⁸ Some of these lines have been shown to secrete M-CSF, which suggests a role for this factor in bone marrow-dependent hemopoiesis.^{20,33} It is apparent that these cell lines are morphologically and functionally diverse; thus they may represent some of the different cell types that constitute the hemopoietic inductive microenvironment.

Our studies with the bone marrow stromal cell clone GY30 examined the production of humoral factors and their relationship to CSFs derived from other sources. These results show that GY30 cells are functionally distinct from previously described stromal cell lines. In addition, we have investigated the possibility that CSF production by GY30 cells is regulated by various stimulants similar to the induction of CSF synthesis by T cells.

MATERIALS AND METHODS

Animals. CBA/J and C3H/HEJ mice (Jackson Laboratories, Bar Harbor, ME), aged 8 to 12 weeks, were used as a source of bone marrow cells.

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Establishment of adherent cell layers. Bone marrow cells obtained by flushing the femurs of CBA/J and C3H/HEJ mice were plated at 1×10^6 cells/mL in modified Iscove's medium, 5×10^{-5} mol/L 2-mercaptoethanol (2-ME) and 10% fetal calf serum (FCS). Cultures established at 37 °C in a humidified atmosphere with 5% CO₂ in air were fed weekly by removing half of the medium and replacing it with fresh medium. After 3 months of continuous culture, adherent cells were trypsinized, washed, and one fifth of the cells were subcultured in new flasks. Cultures usually reformed an adherent layer after 2 weeks, at which time they were repeatedly passaged into new flasks.

Single-cell cloning. A dilute suspension of adherent layer cells was plated in a 100-mm Petri dish. With the aid of an inverted microscope, single cells were micropipetted and transferred to separate wells in a Falcon microtiter tray (Falcon Labware, Oxnard, CA) containing modified Iscove's medium, 2-ME, and 10% FCS. All wells were inspected for growth and clonality of the resulting line. Two clonal lines obtained by this method were used in this study. They are referred to as GY30 and HEJ.2 (derived from CBA/J and C3H/HEJ bone marrow cultures, respectively).

Histochemical and immunofluorescent staining. GY30 cells were removed from culture with a rubber policeman and applied to glass slides using a cytocentrifuge. The mounted cell preparations were stained for myeloperoxidase, chloroacetate esterase, alpha-naphthyl acetate esterase, alkaline phosphatase, and acid phosphatase by established histochemical methods.

GY30 cells were grown in a subconfluent layer in tissue culture chamber slides (Lab-Tek, Miles Scientific, Naperville, IL) for immunofluorescent staining of cell surface determinants. Growth medium was removed, and the adherent cells were overlaid with either anti-Ia^k, anti-H-2K^k, anti-Thy1.2, anti-Ly1, anti-Ly2 (Becton Dickinson, Oxnard, CA), or anti-Mac1 antibodies (gift of Robert Coffman, DNAX). Unrelated antibodies of the same isotypes as the specific monoclonals just mentioned were added to duplicate cultures to control for nonspecific binding. After 15 minutes, the slides were washed and fluorescein isothiocyanate (FITC)-conjugated rat antimouse IgG antibodies (Pel-Freez Laboratories, Rogers, AR) or FITC-conjugated rabbit antirat Ig antibodies (Robert Coffman) were added to the appropriate sample. After 30 minutes, the slides were washed, coverslipped, and examined with a Zeiss fluorescence microscope.

Preparation of conditioned medium. Fresh unsupplemented medium or medium containing various concentrations of phytohemagglutinin (PHA) (Wellcome Diagnostics, Buckenham, England), concanavalin A (Con-A) (Sigma Chemical Co, St Louis), lipopolysaccharide (LPS) (Sigma), phorbol 12-myristate 13-acetate (PMA) (Sigma), P388D1 macrophage supernatant, or recombinant IL-1 were added to confluent layers of GY30 or HEJ.2 cells. At various intervals (12 hours, 24 hours, and four days), the conditioned medium (CM) was collected, filtered through a 0.45-μm millipore membrane, and stored at -20 °C.

To prepare P388D1 CD, P388D1 macrophage cells were grown to a confluent layer in RPMI 1640, 10% FCS, and 5×10^{-5} mol/L 2-ME. The adherent layer was then cultured in fresh medium containing 0.5% FCS. After four days, the supernatant was harvested and stored as described.

Source of factors. Purified G-CSF (4×10^4 U/mL) was a gift of N. Nicola (Walter and Eliza Hall Institute). Purified M-CSF (CSF-1) at 2×10^4 U/mL was a gift of R. Shaduck (University of Pittsburgh School of Medicine). Purified recombinant IL-1 (10^6 U/mL) was a gift of P. Lomedico (Hoffman-La Roche, Nutley, NJ). Recombinant IL-2, IL-3, and GM-CSF were obtained from Cos 7 cells transfected with the corresponding cDNA clones as previously described.³⁹⁻⁴¹ One unit of IL-2, IL-3, G-CSF, M-CSF, or GM-CSF is defined as that amount of factor that stimulates

half-maximal [³H]-thymidine incorporation of factor-dependent lines cultured at 5×10^4 cells/mL.

Factor-dependent cell lines. MC/9 mast cells⁴² were grown in RPMI 1640, 5×10^{-5} mol/L 2-ME, and 10% FCS supplemented with recombinant IL-3. NFS-60 and DA 3 myeloid cell lines (both provided by J. Ihle, Frederick Cancer Research Institute) were subcloned by micromanipulation. A GM-CSF-responsive subclone of DA 3 cells, referred to as DA 3.15, was isolated and has been continuously grown for 6 months in basic medium supplemented with recombinant GM-CSF. A subclone derived from NFS-60 cells, referred to as NFS-60.4, has been maintained in basic medium and recombinant IL-3. The T cell line HT2 (supplied by S. Strober, Stanford University) was cultured in complete medium containing recombinant IL-2.

Proliferation assays. Factor-dependent cells were cultured at 5×10^3 cells/well (Falcon microtiter trays) in RPMI 1640, 4% FCS, 5×10^{-5} mol/L 2-ME, and varied concentrations of test supernatants. During the last six hours of a 24-hour incubation period, each culture was pulsed with 0.5 μCi [³H]-thymidine. The cells were harvested onto glass fiber filters, and the radioactivity was measured in a liquid scintillation spectrometer.

Spleen colony-forming unit assay. The CFU-S content of cultured or fresh bone marrow cells was assayed by the method of Till and McCulloch.⁴³ Each lethally irradiated (900 rad) CBA/J mouse was injected intravenously with one fifth of the nonadherent bone marrow cells harvested from a single culture flask that had been seeded with 5×10^5 nonadherent bone marrow cells. Control groups were injected with 10^5 fresh bone marrow cells or received no cells. After ten days, the spleens (five mice per group) were removed and fixed in Bouin's solution and the splenic nodules counted using a dissecting microscope. The average background spleen colony count in mice not injected with cells was <0.5 per spleen. The average count in the control group receiving 10^5 uncultured bone marrow cells was 17 ± 4 .

Bone marrow colony-forming assay. Bone marrow cells were suspended at 1×10^6 cells/mL in RPMI 1640 with 2% FCS and incubated in tissue culture-grade Petri dishes for two hours. The nonadherent cells were harvested and cultured at 10^5 cells/mL in 35-mm Petri dishes containing modified Iscove's medium, 25% FCS, 5×10^{-5} mol/L 2-ME, 0.8% (wt/vol) methylcellulose using either IL-3, GM-CSF, or GY30 CM as a source of growth factor. After three days of culture, 1 U/mL erythropoietin (Step III, Terry Fox Laboratories, Vancouver, BC, Canada) was added to one set of duplicate cultures. Colonies consisting of more than 50 cells were counted on day 7. Individual colonies were applied to glass slides and stained with Wright-Giemsa for morphological analysis.

Endotoxin determination. Purified recombinant IL-1 was assayed for the presence of endotoxin using the limulus amoebocyte lysate test (E-toxate kit, Sigma). IL-1 prepared at 10, 1, and 0.1 U/mL was compared with various concentrations of an *Escherichia coli* endotoxin reference (Lot EC-1) according to the E-toxate procedure. The limit of detection of endotoxin is 0.05 to 0.2 ng/mL.

Preparation of mRNA and RNA blot analysis. GY30 cells were harvested from flasks by treatment with trypsin and then lysed with guanidine thiocyanate according to the procedure of Chirgwin et al.⁴⁴ The total cellular RNA was isolated by centrifugation through CsCl and recovered by ethanol precipitation. PolyA⁺ RNA was isolated following oligo (dT) cellulose chromatography.⁴⁵ As a control, mRNA was also isolated from the helper T cell clone Cl.Ly1+2-/-⁴²

For RNA blot analysis, polyA⁺ RNA was denatured and treated with formaldehyde as described by Maniatis et al.⁴⁶ and then fractionated on a 1% agarose gel containing formaldehyde. The RNA was then transferred to a nylon/nitrocellulose filter (Nytran, Schleicher and Schuell, Keene, NH). Filters were prehybridized and

hybridized in $6\times$ SSPE containing 50% formamide⁴⁷ at 55 °C. The GM-CSF probe was made using a *Bam*HI fragment containing a full-length mouse GM-CSF cDNA⁴⁰ subcloned into the GEM-1 vector (Promega Biotec, Madison, WI) that carries the SP6 promoter. ³²P-labeled RNA probe was synthesized in vitro using SP6 RNA polymerase and ³²P-cytidine triphosphate or ³²P-guanosine triphosphate essentially as described by Melton et al.⁴⁸ The G-CSF probe was made by fill-in labeling of synthetic oligonucleotides corresponding to residues 68 to 163 and 599 to 694 of the mouse G-CSF cDNA clone.⁴⁹ Following overnight hybridization, RNA blots were washed in $0.1\times$ SSPE at 65 °C and then exposed to x-ray film.

Biosynthetic labeling and analysis of extracellular matrix proteins. Cell lines were labeled with 50 μ Ci/mL of [³H]-proline (100 Ci/mmol; Amersham Corp, Arlington Heights, IL) in 5 mL of RPMI medium supplemented with 100 μ g/mL ascorbate, 64 μ g/mL 2-amino-propionitrile fumarate, and 5% dialyzed FCS. Cells were labeled for 24 hours, and the medium was harvested. The following were then added to the medium: EDTA (20 mmol/L), N-ethyl maleimide (10 mmol/L), and phenylmethyl sulfonylfluoride (1 mmol/L). The proteins were precipitated by the addition of solid ammonium sulfate to a 45% saturation followed by stirring overnight at 4 °C. The precipitated protein was collected by centrifugation, then the pellet was resuspended in and dialyzed extensively against 0.03 mol/L Tris-HCl, pH 7.8, 2 mol/L urea, 0.1% Triton X-100, and 0.02 mol/L EDTA. Aliquots of the labeled proteins were then treated with pepsin or collagenase essentially as described by Zipori et al.⁵⁰ and analyzed under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 5% gels.⁴⁷ [³H]-labeled type I and type IV collagen standards were kindly provided by Dr B. Blumberg (University of California, Los Angeles).

Assay of angiotensin-converting enzyme activity. Angiotensin-converting enzyme activity was assayed by a spectrophotometric method using the ACE kit purchased from Sigma. GY30 cell lysate from 10^6 cells was prepared by adding 3 mL of 0.05 mol/L HEPES buffer, pH 8.0, containing 0.15 mol/L NaCl and 1% vol/vol Triton X-100. After 30 minutes the cells were removed from the flask with a rubber policeman and homogenized. Aliquots of GY30 homogenate and angiotensin-converting enzyme standard were assayed for activity according to the ACE kit procedure.

Electron microscopy. GY30 cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, containing 0.05% CaCl₂. The fixative was replaced with 0.1 mol/L cacodylate buffer with 0.05% CaCl₂ and 7% wt/vol sucrose. Cells were postfixated with 1% OsO₄, dehydrated in an ascending ethanol series, and then embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined and photographed with a Philips EM 201 electron microscope.

RESULTS

Derivation and morphological characterization of cloned stromal cell lines. An adherent layer of CBA/J bone marrow cells was established in long-term culture for 3 months with weekly feeding. During this period a steady production of nonadherent hemopoietic cells was sustained. Morphological examination of the nonadherent population revealed maturing neutrophilic granulocytes and macrophages. After the initial 3-month culture period, nonadherent cells were discarded, and the adherent cells were passaged every other week. One of the subcultures was able to quickly reform a confluent layer of cells and could be readily cloned by micromanipulation. Twenty-two clones were suc-

cessfully derived from 182 micropipetted cells. Neither the adherent layers nor the resulting cloned lines require exogenous factors for growth, except those contained in FCS.

One clone, designated GY30, survived more than 20 passages and was selected for further study. Although GY30 cells do not display density-dependent growth typical of primary cell cultures, they cease growing in vitro after reaching confluency and do not form tumors when injected into normal or sublethally irradiated (400 rad) CBA/J mice. In vitro, this clone grows with an approximate doubling time of 30 to 48 hours and consists of large flat cells (Fig 1A) that form a confluent monolayer with a cobblestone appearance. If cultures are allowed to become confluent for 8 weeks or longer, approximately 3% of the cells spontaneously accumulate lipid vacuoles (Fig 1B). Lipid accumulation has been observed in preadipocytes that may be of fibroblastoid or epithelial cell origin. Although the significance of adipogenesis is not clear, it seems to be related to growth arrest.⁵¹ We have also found that GY30 cells are myeloperoxidase-, acid phosphatase- and nonspecific esterase-negative but chloroacetate esterase- and alkaline phosphatase-positive. Further, they have the following surface phenotype: H-2+, Thyl+,

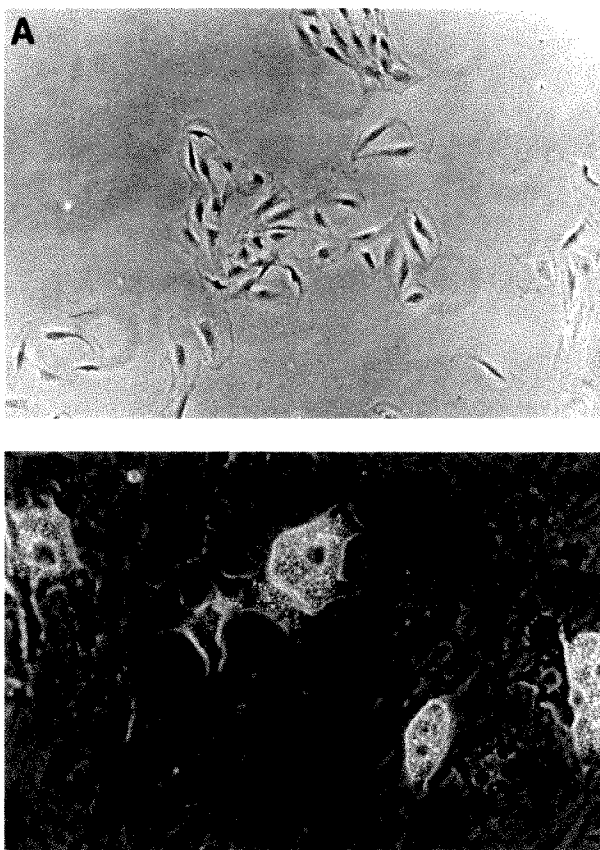


Fig 1. Phase-contrast micrographs of GY30 adherent cells. (A) Actively growing cultures. (Original magnification $\times 300$; current magnification, $\times 96$. (B) Eight-week-old stationary cultures wherein approximately 3% of GY30 cells have undergone adipogenesis. (Original magnification $\times 300$; current magnification $\times 102$.)

Ia⁺, Lyl⁺2⁺, Mac1⁺. These results indicate that GY30 cells are not macrophages.

Analysis of extracellular matrix proteins biosynthetically labeled with [³H]-proline revealed that GY30 cells synthesize predominantly type I collagen along with small amounts of collagen types IV and V (Fig 2). This profile of collagen production suggests that GY30 cells share properties with both fibroblasts and endothelial cells.⁵² Electron micrographs of GY30 cells failed to demonstrate Weibel-Palade bodies, which have been identified as specific components of arterial endothelial cells *in situ*.⁵³ Further, the endothelial origin of GY30 cells could not be established by the presence of angiotensin-converting enzyme activity.^{54,55} Thus, GY30 cells are tentatively classified as fibroendotheliallike cells.

Using the same methodology, several stromal cell lines were cloned from an established adherent layer of C3H/HEJ bone marrow cells. The line referred to as HEJ.2 had properties similar to GY30 cells, and its use in certain experiments will be described later.

Effects of GY30 adherent layer and GY30 CM on CFU-C

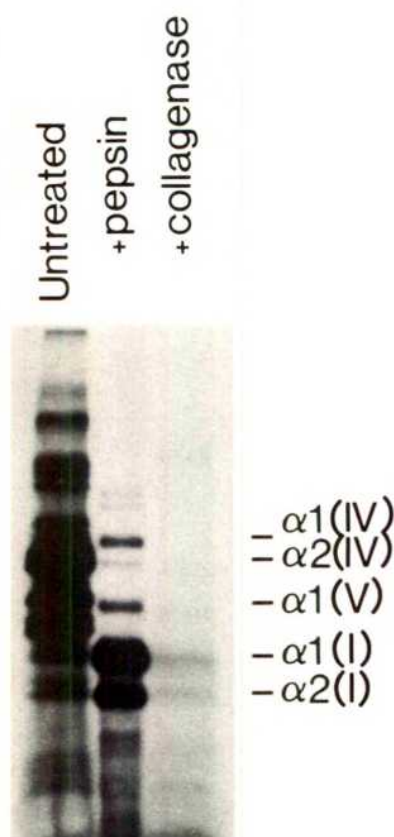


Fig 2. Analysis of [³H]-proline-labeled extracellular proteins. GY30 cells were incubated with [³H]-proline as described in Materials and Methods. Proteins in the medium were concentrated by ammonium sulfate precipitation and then analyzed by polyacrylamide gel electrophoresis following treatment with pepsin or collagenase. The position of collagen standards is indicated. The gel was fixed and then soaked in a fluorographic solution (Enlightning, New England Nuclear, Boston) prior to autoradiography.

and CFU-S production. Adherent cell-depleted bone marrow cells (5×10^5) were seeded on top of a confluent layer of GY30 cells or cultured in 30% GY30 CM. At weekly intervals, two identical cultures from each set were terminated, and all of the nonadherent cells were harvested by gently washing the bottom of the flask. The total cell production and CFU-C and CFU-S content were determined at each time point. The number of CFU-C and CFU-S present in the uncultured nonadherent bone marrow cells was assayed as described in Materials and Methods. It was calculated that 5×10^5 fresh bone marrow cells contained 85 CFU-S and 1.15×10^3 CFU-C when cultured in IL-3.

The total number of viable cells recovered from cultures supplemented with GY30 CM rapidly declined after the second week of culture, whereas a steady production of mature neutrophilic granulocytes and macrophages was maintained in cultures containing GY30 adherent layers (Fig 3A). Moreover, cells recovered from cultures supplemented with GY30 CM failed to form hemopoietic colonies when plated in semisolid culture medium with IL-3 and erythropoietin (Fig 3B). However, a small but significant number of colony-forming cells (CFU-C) were detected for many weeks in cultures where hemopoietic cells were in direct contact with the GY30 cell layer. The colonies

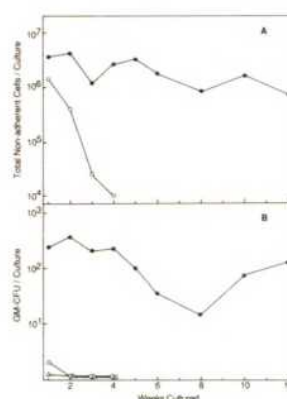


Fig 3. Hemopoietic cells recovered from long-term cultures containing GY30 adherent cells or GY30 CM. (A) Results are expressed as the mean number of nonadherent cells harvested from duplicate cultures containing GY30 adherent layers (●—●) or only GY30 CM (○—○). Each culture flask contained 5×10^5 nonadherent cells in a total volume of 5 mL. The adherent layer cultures were fed biweekly by removing half of the medium and replacing it with fresh unsupplemented medium, and the other set of cultures were replenished with GY30 CM at 48-hour intervals. Any cells removed during the exchange of medium were added back to the respective cultures. Control cultures contained 5×10^5 nonadherent cells plated in the absence of an adherent layer or CM. After 1 week of incubation, there were no viable cells detected in these control cultures. (B) Results are expressed as the mean number of colony-forming cells (GM-CFU) recovered from duplicate cultures harvested at each time point; GY30 adherent layers (●—●), GY30 CM (○—○), and unsupplemented medium (△—△). To enumerate GM-CFU, nonadherent cells from each culture were divided between three 35-mm dishes containing semisolid growth medium supplemented with 1,000 U/mL of IL-3 and 1 U/mL of erythropoietin as described in the text. The resulting colonies consisting of greater than 50 cells were scored after seven days of incubation.

detected in the CFU-C assay consisted entirely of neutrophilic granulocytes and macrophages.

At the same time points, cells harvested from identical parallel cultures were tested for CFU-S content. We found that they failed to form spleen colonies when injected into lethally irradiated mice (data not shown). These results suggest that GY30 cells fail to support pluripotential (CFU-S) whereas they are capable of maintaining a fraction of the granulocyte-macrophage progenitor cells (GM-CFU) present in the original input nonadherent bone marrow population. Alternatively, GY30 cells may support a small number of pluripotential cells that are undetectable by the CFU-S assay, but these stem cells give rise to a modest number of GM-CFU during long-term culture. Our data do not discern between these possibilities.

Production of colony-stimulating activity. The colony-stimulating activity of GY30 CM collected four days after the last medium change was evaluated in conventional semisolid bone marrow cultures. The results presented in Table 1 show that GY30 CM stimulates a significant number of nonerythroid colonies compared with IL-3 and GM-CSF. Despite the presence of erythropoietin, no pure or mixed erythroid colonies above background levels were observed. When the cellular composition of individual colonies was examined, they were found to consist entirely of neutrophilic granulocytes, macrophages, or a mixture of the two cell types.

Characterization of the colony-stimulating activities using factor-dependent cell lines and cDNA probes. To further characterize the colony-stimulating activities pro-

duced by GY30, supernatant was collected four days after the addition of fresh medium and was examined in 24-hour proliferation assays using factor-dependent cell lines. The cell lines selected for this study have been characterized with respect to their dependence on several defined growth factors (Fig 4A to D). Purified macrophage colony-stimulating factor (M-CSF) does not stimulate any of these cell lines. Only HT2 T cells responded to recombinant IL-2. The mast cell line MC/9 and the myeloid cell lines NFS 60.4 and DA 3.15 are all stimulated to proliferate by recombinant IL-3. However, NFS 60.4 and DA 3.15 cells are also stimulated by purified G-CSF and recombinant GM-CSF, respectively. Thus, the pattern of response of this cell panel was used to functionally identify as well as quantitate growth factor activities present in test supernatants.

We found that GY30 CM (day 4) fails to stimulate proliferation of IL-2-dependent HT2 T cells (Fig 4A) and IL-3-dependent MC/9 mast cells (Fig 4B). However, it does support proliferation of NFS 60.4 cells (Fig 4C) and DA 3.15 cells (Fig 4D). Taken together, these data show that GY30 stromal cells produce colony-stimulating activities distinct from IL-2, IL-3, and M-CSF but functionally similar to GM-CSF and G-CSF.

These data do not rule out the possibility that GY30 cells also produce M-CSF. However, the low frequency of macro-

Table 1. Colony-Stimulating Activity of GY30 CM

Source of CSF	EPO (1 Unit per Culture)	Number of Colonies/ 10^5 BM Cells*	
		Nonerythroid	Containing Erythrocytes
GY30 CM			
30%	+	71 $\pm$ 5†	5 $\pm$ 2
	—	79 $\pm$ 10	0
3%	+	11 $\pm$ 4	3 $\pm$ 2
0.3%	+	5 $\pm$ 2	2 $\pm$ 1
GM-CSF (500 U/mL)	+	126 $\pm$ 32	12 $\pm$ 3
	—	119 $\pm$ 17	0
IL-3 (500 U/mL)	+	216 $\pm$ 28	52 $\pm$ 9
	—	201 $\pm$ 42	0
None (Medium control)	—	4 $\pm$ 2	3 $\pm$ 2
	—	7 $\pm$ 3	0

Nonadherent bone marrow (BM) cells were suspended at 10^5 cells in 1 mL of methylcellulose culture medium supplemented with various concentrations of GY30 CM. Control cultures contained IL-3 or GM-CSF as a source of growth factor. After three days of incubation, 1 unit of erythropoietin (EPO) was added. On the seventh day, the resulting colonies consisting of 50 or more cells were counted.

*Data from triplicate cultures are expressed as means $\pm$ SEM.

†All of the colonies from two cultures were picked, applied to glass slides, and analyzed after staining fixed cells with Wright-Giemsa. The cellular composition of individual colonies was as follows: 58% granulocyte, 7% macrophage, and 35% mixed granulocyte/macrophage.

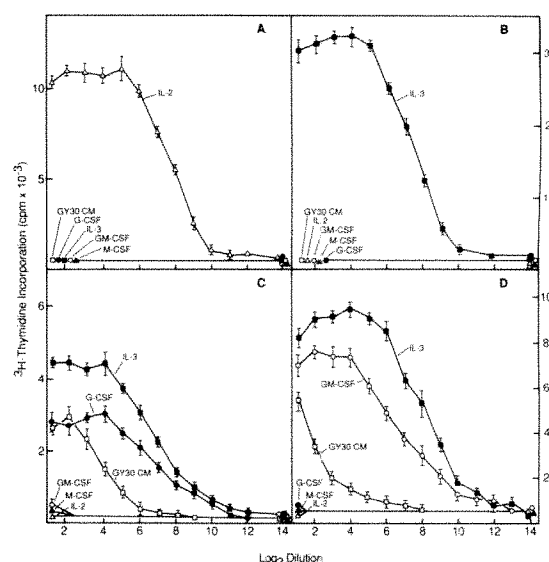


Fig 4. [^{3} H]-thymidine incorporation of factor-dependent cell lines stimulated by defined growth factors and GY30 CM. A quantity of 5×10^3 factor-dependent cells per culture (0.1 mL total volume) were incubated for 24 hours in growth medium supplemented with varying concentrations of IL-3 (■—■), IL-2 (▲—▲), GM-CSF (○—○), G-CSF (●—●), M-CSF (△—△), or day 4 GY30 CM (□—□). Each culture was pulsed with [^{3} H]-thymidine during the last six hours of incubation and harvested onto glass fiber filters and the radioactivity determined. Titrations of GY30 CM and various stock solutions of defined growth factors (adjusted to approximately 1,000 U/mL) were started at 1:2 dilutions in the first well. Results of triplicate cultures are expressed as the mean counts per minute $\pm$ SEM. (A), HT2 T cells; (B) MC/9 mast cells; (C) NFS 60.4 cells; (D) DA 3.15 cells.



Fig 5. RNA blot analysis of GY30 mRNA probed with a GM-CSF cDNA probe. mRNA from the (A) T cell clone Cl.Ly 1+2-/9 (5 µg) or from (B) GY30 cells (10 µg) was run on a 1% agarose-formaldehyde gel and then blotted to a Nytran nylon/nitrocellulose membrane. The filter was hybridized with a ^{32} P-labeled GM-CSF probe prepared as described in Materials and Methods. The autoradiogram was exposed for three days at -70°C using an intensifying screen.

phage colonies observed in the CFU-C assay (Table 1) indicates that, if present, the concentration may be limiting.

GY30 mRNA was analyzed by RNA blot hybridization using molecular probes for several growth factors. The probe for GM-CSF hybridized to a mRNA species that migrated at the same position as the GM-CSF band in a control RNA sample derived from Cl.Ly1+2-/9 T cells (Fig 5). This result clearly establishes that GY30 cells produce GM-CSF. When GY30 mRNA was analyzed with probes for mouse G-CSF, no hybridization could be detected, although later studies (described later) suggest that low levels of G-CSF mRNA are made that are below the level of detection by RNA blotting. In agreement with the results of bioassays of the CM, no hybridization could be detected using probes for IL-2 or IL-3 (data not shown).

LPS stimulation of CSF production. We then wished to determine whether CSF production by GY30 cells could be regulated by various stimulating agents known to activate other lymphoid or hemopoietic cells. In these studies we tested the effects of various concentrations of PHA, PMA, Con-A, and LPS. CM from treated and untreated GY30 cells were collected after 12 hours and tested using the four factor-dependent cell line already described.

Treatment with PMA, Con-A, or PHA had no effect on GY30 cells in any of the assays (data not shown). In contrast, LPS treatment of GY30 cells stimulated increased activity detected by the G-CSF-responsive cell line NFS 60.4 (Fig 6). A total of 20 ng/mL LPS was required to give maximum production of the G-CSF-like activity, and concentrations of 0.02 ng/mL or less had no effect. We calculated that 70 U/mL of G-CSF activity was present in stimulated GY30 supernatant compared with less than 1 U/mL in unstimulated 12-hour supernatant. One unit was defined as the amount of factor that induced half-maximal stimulation of ^3H -thymidine uptake by NFS 60.4 cells under the assay conditions described. LPS titrated into NFS 60.4 cultures at a starting concentration of 200 ng/mL had no effect by itself. Moreover, the addition of various concentrations of LPS to unstimulated 12-hour supernatant did not result in enhanced stimulation of NFS 60.4 cells.

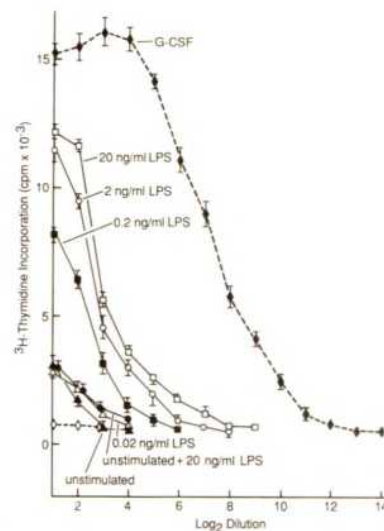


Fig 6. ^3H -thymidine incorporation of NFS 60.4 cells stimulated by supernatants of LPS-treated GY30 cells. A total of 5×10^3 NFS 60.4 cells per culture (total volume of 0.1 mL) were incubated in medium supplemented with varying concentrations of GY30 supernatants induced for 12 hours with LPS at 20 ng/mL ($\square$ — $\square$), 2 ng/mL ($\circ$ — $\circ$), 0.2 ng/mL ($\blacksquare$ — $\blacksquare$), and 0.02 ng/mL ($\triangle$ — $\triangle$) or unstimulated 12-hour GY30 supernatant ($\blacktriangle$ — $\blacktriangle$) or unstimulated GY30 supernatant containing 20 ng/mL LPS ($\bullet$ — $\bullet$). NFS 60.4 cells were also cultured with varying concentrations of purified G-CSF ($\diamond$ — $\diamond$) and LPS ($\diamond$ — $\diamond$). Stock solutions contained 1,000 U/mL G-CSF and 200 ng/mL LPS. Cell cultures were harvested as described in the legend to Fig 4. Results of triplicate cultures are expressed as mean counts per minute $\pm$ SEM.

Our ability to assess the GM-CSF activity of LPS-induced GY30 supernatants was complicated by the fact that certain concentrations of LPS alone directly stimulate proliferation of the DA 3.15 cell line. LPS at concentrations of 2 ng/mL or less does not stimulate DA 3.15 cells, but 2 ng/mL of LPS does stimulate increased production of GM-CSF by GY30 cells (detected by proliferation of DA 3.15 cells, Fig 7). The residual LPS in this sample does not appear to mediate this effect since 2 ng/mL LPS added to an unstimulated supernatant did not cause increased proliferation of the target cells (Fig 7). Stimulation of GY30 cells with 2 ng/mL LPS induced approximately 40 U/mL of GM-CSF, whereas untreated cells produced less than 1 U/mL. None of the supernatants from LPS-treated GY30 cells stimulated IL-2-dependent T cells (HT-2) or IL-3-dependent mast cells (MC/9) (data not shown). Thus, LPS induces increased production of GM-CSF and an activity similar to G-CSF. However, it does not induce new expression of IL-2- or IL-3-like activities.

Recombinant IL-1 enhances CSF production. To determine whether macrophages and T cells secrete molecules that regulate stromal cell function, GY30 cells were cultured with CM of various T cell lines and the macrophage cell line P388D1. Increased levels of CSF activity were found only in supernatants of GY30 cells incubated with P388D1 CM (data not shown). This result raised the possibility that a macrophage product such as IL-1 mediates this effect. Therefore, GY30 monolayers were cultured with varying

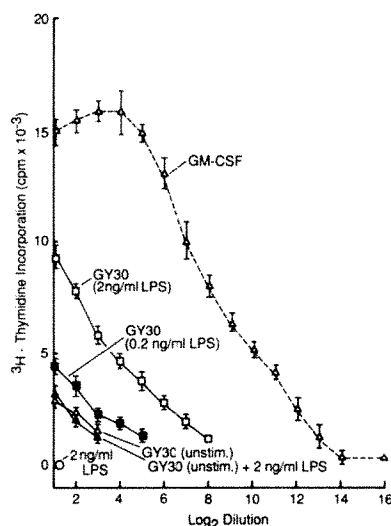


Fig 7. [^3H]-thymidine incorporation of DA 3.15 cells stimulated by supernatants of LPS-treated GY30 cells. The general culture conditions were described in the legend to Fig 4. DA 3.15 cells were cultured with varying concentrations of supernatants of GY30 cells treated with LPS at 2 ng/mL ($\square$ — $\square$) and 0.2 ng/mL ($\blacksquare$ — $\blacksquare$), supernatant of unstimulated GY30 cells (Δ — Δ), and unstimulated GY30 supernatant containing 2 ng/mL LPS ($\blacktriangle$ — $\blacktriangle$). DA 3.15 cells were also cultured with 2 ng/mL LPS ($\circ$ — $\circ$) and varying concentrations of recombinant GM-CSF (Δ — Δ). Results of triplicate cultures are expressed as mean counts per minute $\pm$ SEM.

concentrations of recombinant IL-1. Supernatants were harvested after 12 hours and tested in a [^3H]-thymidine incorporation assay using the factor-dependent cell lines. We found that supernatants of GY30 cells treated with 10 U IL-1/mL or more contained at least 210 U/mL of the G-CSF activity (Fig 8A, measured by NFS 60.4 cells) and 230 U/mL of GM-CSF (Fig 8B, measured by DA 3.15 cells). IL-1 at various concentrations had no effect on these cells by itself. Further, the addition of 10 U IL-1/mL to uninduced GY30

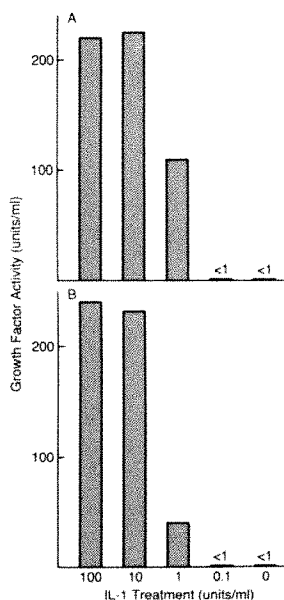


Fig 8. IL-1 stimulates increased CSF production by GY30 stromal cells. (A) NFS 60.4 and (B) DA 3.15 cells were cultured with supernatants of GY30 cells treated for 12 hours with 0, 0.1, 1, 10, or 100 U/mL recombinant IL-1. The levels of [^3H]-thymidine incorporation stimulated by these various supernatants and purified G-CSF or GM-CSF were assayed as described in the legend to Fig 4. The stimulatory activities of IL-1-induced GY30 supernatants are expressed in units per milliliter where 1 unit is defined as the amount of factor that stimulates half-maximal [^3H]-thymidine incorporation of the factor-dependent cells.

supernatant did not result in enhanced stimulation of the factor-dependent cell lines. We also found that IL-1-induced GY30 supernatants failed to stimulate HT2 T cells and MC/9 mast cells (data not shown).

These results suggest that IL-1 as well as LPS may up-regulate the production of colony-stimulating activities by the stromal cell line. However, it was necessary to rule out that the induction by IL-1 was due to traces of endotoxin contamination. Since IL-1 added directly into DA 3.15 cell cultures had no effect by itself, we reasoned that any endotoxin contamination must be 2 ng/mL or less. This concentration was already shown to be nonstimulatory for this LPS-responsive cell line (Fig 7). This conclusion was confirmed when IL-1 prepared at 10 U/mL was negative using the limulus amoebocyte lysate test (endotoxin sensitivity of 0.05 to 0.2 ng/mL). Additional evidence was obtained with the stromal cell line HEJ.2 derived from LPS-resistant C3H/HEJ mice. HEJ.2 cells share many properties with GY30 cells including increased production of G-CSF-like activity in response to 10 U/mL IL-1 (Fig 9B, measured by NFS 60.4 cells). In this case, due to the LPS-resistant nature of these cells, LPS concentrations in excess of 3 $\mu\text{g}/\text{mL}$ were required to induce any detectable CSF production within the same 12-hour culture period (Fig 9A). The possibility that the IL-1 preparation was contaminated with these levels of endotoxin was already ruled out by two other means (LPS sensitivity of DA 3.15 cells and the limulus amoebocyte lysate test).

Regulation of CSF mRNA levels by IL-1. mRNA was isolated from GY30 cells 12 hours following the addition of IL-1 and was compared in a RNA blot with mRNA from untreated cells. The results in Fig 10 show that the levels of GM-CSF mRNA are highly inducible by IL-1. The induced mRNA appears to be identical in length to the mRNA produced by uninduced cells or by T cells. These results strongly suggest that IL-1 directly induces new transcription of GM-CSF mRNA in GY30 cells.

We also analyzed GY30 mRNA for G-CSF transcripts using G-CSF cDNA probes. Although no detectable hybridization was found in the mRNA from untreated GY30 cells, mRNA from IL-1-induced cells showed a distinct band of hybridization at approximately the same size previously reported for G-CSF transcripts (1.7 kb).⁴⁹ Based on these results, we believe it is likely that uninduced GY30 cells

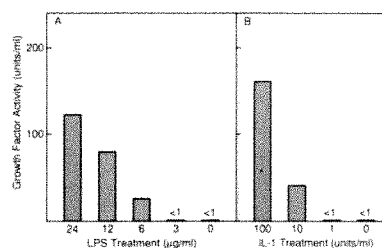


Fig 9. LPS and IL-1 stimulate CSF production by HEJ.2 stromal cells. NFS 60.4 cells were cultured with supernatants of HEJ.2 cells treated with (A) 3, 6, 12, and 24 $\mu\text{g}/\text{mL}$ LPS and (B) 1, 10, and 100 U/mL recombinant IL-1. The growth factor activity of the HEJ.2 supernatants, expressed as units per milliliter, was assayed as described in the legend to Fig 4.

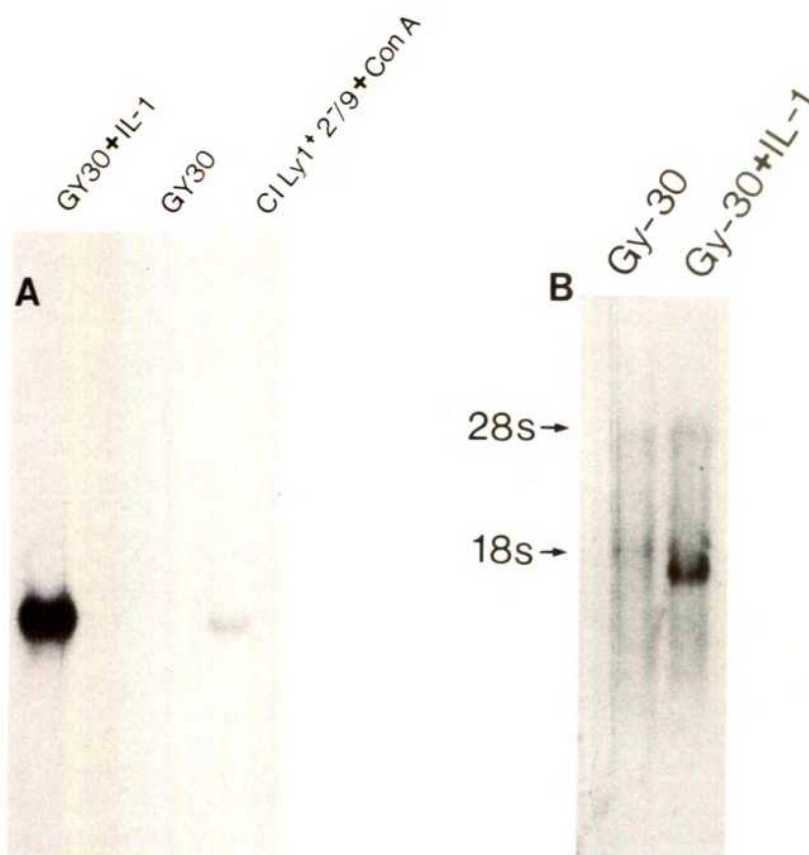


Fig 10. (A) Induction of GM-CSF mRNA in GY30 cells by IL-1. mRNA was isolated from untreated GY30 cells or from cells 12 hours following the addition of 100 U/mL of recombinant IL-1. RNA blot analysis was carried out as described in Materials and Methods by using a GM-CSF probe. The autoradiogram was exposed for 16 hours at -70°C using an intensifying screen. The GM-CSF band in uninduced GY30 mRNA requires longer exposure times to be detectable. (B) Induction of G-CSF mRNA in GY30 cells by IL-1. mRNA samples isolated as in (A) were analyzed in RNA blots using two ^{32}P -labeled synthetic oligonucleotides corresponding to residues 68 to 163 and 599 to 694 of the mouse G-CSF cDNA.

produce low levels of G-CSF mRNA undetectable by RNA blotting that account for the NFS 60.4-stimulatory activity.

DISCUSSION

Stromal cells produce GM-CSF and G-CSF. Studies using clonal lines of stromal cells provide a powerful means to understand the mechanisms by which the bone marrow microenvironment regulates stem cell growth and differentiation. Important questions arise as to the nature of the signals mediating interactions between stromal cells and hemopoietic progenitor cells, including the possible production of soluble factors and their relationship to hemopoietic regulators made by cells outside the marrow such as T lymphocytes. Although previous studies have shown that several stromal cell clones produce M-CSF,^{20,33} a few lines apparently secrete additional granulocyte and macrophage colony-stimulating activities.³⁸ Neither the number of factors involved nor their precise identity could be established.

We describe here the characterization of a fibroendothelial-like stromal cell clone, GY30, which was isolated from the adherent layers of bone marrow cultures. GY30 cells sustain through direct contact the production of committed granulocyte-macrophage progenitor cells (GM-CFU) but fail to support the survival of multipotential stem cells as detected by the CFU-5 assay. Further, the supernatant of GY30 cells stimulates the formation of granulocyte-macrophage colonies in semisolid bone marrow cultures. Using a panel of factor-dependent cell lines, we show that no significant levels of IL-3 or IL-2 could be detected in GY30 cell supernatants.

However, results with these cell lines suggest that GY30 cells produce activities similar to GM-CSF and G-CSF. Both factors could be unambiguously demonstrated by the presence of GM-CSF and G-CSF transcripts detected using specific cDNA probes. These results show that GM-CSF, which is made by activated T cells, also plays a role in controlling steady-state hemopoiesis in the bone marrow. Our results also establish that G-CSF, previously shown to be made by peritoneal cells⁵⁶ and by a fibrosarcoma cell line⁴⁹ must also be involved in marrow hemopoiesis.

Production of CSFs by stromal cells is modulated by LPS and IL-1. We provide the first evidence that the production of hemopoietic growth factors by marrow stroma in vitro can be modulated by external factors; the production of both GM-CSF and G-CSF by GY30 cells can be greatly enhanced (70- to 200-fold) by LPS and the cytokine IL-1. Parallels can be drawn between the inducible production of CSFs by bone marrow stromal cells and the inducible expression of CSFs by T cells activated by either antigen or mitogens. In both stromal cells and T cells, the induction of factors is correlated with increased levels of the corresponding mRNAs.

The action of LPS, which we document in vitro, suggests a role for this factor in regulating hemopoietic factor production in vivo. Under steady-state conditions, the bone marrow produces a constant number of various blood cells, but it is not clear what regulatory signals establish the balance between stem cell self-renewal and the maturation of progenitor cells. Nevertheless, it is known that the number of

circulating neutrophilic granulocytes is increased in mice following the administration of bacterial products such as endotoxin. Under these conditions, there are also elevated serum levels of granulocyte-macrophage colony-stimulating activities^{57,58} as well as granulocytic hyperplasia of the bone marrow.⁵⁹ These prior studies suggest that the steady-state production of granulocytes and macrophages by bone marrow can be upregulated in response to bacterial infections. Although it is unclear whether bone marrow is exposed to antigen during most bacterial infections, our results demonstrate that hemopoietic factor production by a bone marrow-derived cell can be augmented directly by the bacterial product, LPS.

The regulatory effects of IL-1 on the stromal cell clone are also consistent with previous studies. In humans, an unidentified factor produced by mononuclear phagocytes was shown to stimulate the production of CSF activities by fibroblasts⁶⁰ and vascular endothelial cells.⁶¹ Because IL-1 production by macrophages and possibly other cell types is elicited by a wide variety of agents, the functional activation of stromal cells by IL-1 could be part of an important

protective mechanism against many types of infection. Furthermore, IL-1 or IL-1-like molecules may also function as normal regulatory signals within the marrow microenvironment. Pure stromal cell populations apparently secrete hemopoietic factors constitutively under standard in vitro culture conditions, but it is not known whether they do so in vivo. It is possible that within the bone marrow microenvironment, stromal cells require endogenous signals such as IL-1 to carry out some of their normal hemopoietic regulatory activities. Thus, all levels of blood cell production might be determined by positive and negative regulation of stromal cell activities.

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Oral Contraceptives and Gender Affect Protein S Status

By L.M. Boerger, P.C. Morris, G.R. Thurnau, C.T. Esmon, and P.C. Comp

Protein S is a plasma protein that serves as a cofactor for the anticoagulant effects of activated protein C. Congenital protein S deficiency is often associated with thromboembolic disease. During pregnancy a decrease in the functional and antigenic levels of protein S occurs; this change in protein S status may contribute to the thromboembolic complications that sometimes occur during pregnancy. In certain patients, oral contraceptive use has also been associated with thrombotic complications. In this study, protein S status was determined in 21 women taking oral contraceptives and compared with that of 21 women not taking oral contraceptives and that of 21 men. The results show that women taking oral contraceptives have significantly lower total protein S ($24.3 \pm 3.6 \mu\text{g/mL}$; mean $\pm$ SD) than women not taking oral contraceptives (28.6 ± 3.9

$\mu\text{g/mL}$) ($P < .005$). Men had significantly higher protein S levels ($30.9 \pm 3.9 \mu\text{g/mL}$, $P < .01$) than age-matched women not taking oral contraceptives. In plasma, an equilibrium exists between free (functionally active) protein S and protein S complexed to C4b-binding protein, which is functionally inactive. The mean levels of C4b-binding protein were essentially the same among the three groups, but the levels of free protein S were significantly different and reflected different total protein S antigen levels. Additionally, we found that inflammation significantly elevated C4b-binding protein levels and could result in a further significant decrease in free protein S levels. These data indicate that plasma protein S levels are significantly affected by hormonal status and inflammation.

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PROTEIN S is a plasma protein that serves as a cofactor for the anticoagulant effects of activated protein C.¹ In this regard, two observations have been reported: (1) congenital protein S deficiency is associated with recurrent thromboembolic disease²⁻⁴ and (2) a relative protein S deficiency occurs in women during pregnancy.⁵ During pregnancy, functional protein S levels are significantly lower ($38\% \pm 17\%$; mean $\pm$ SD) than those found in age-matched, non-pregnant women ($97\% \pm 32\%$; $P < .001$).⁵ The reduced functional protein S levels are accompanied by a significant reduction in total protein S antigen to $68\% \pm 11\%$ ($P < .001$). Based on these data, we postulated that these changes in protein S status are among the factors that predispose pregnant women to thromboembolic complications.

Since oral contraceptive (OC) use hormonally mimics pregnancy and has been associated with thrombotic complications,⁶ we have evaluated OC users to determine whether significant changes in protein S status occur. Since sex hormonal differences could affect protein S levels, we have evaluated the protein S status of healthy males as well.

In normal individuals approximately 40% of protein S is found free in plasma, and 60% is complexed to C4b-binding protein (C4b-BP), an inhibitor of the complement system.⁷

The free protein S is functionally active, and the complexed form is inactive.^{8,9} Because changes in the C4b-BP level may affect the distribution of protein S in plasma, C4b-BP levels along with free protein S and total protein S antigen levels were measured.

MATERIALS AND METHODS

Immunologic assays. Total protein S antigen and free protein S assays were performed as previously described.^{5,8} Purified protein S, prepared as previously described,³ was used to construct a standard curve. Crossed immunoelectrophoresis⁸ was used to estimate the free protein S in the patients with acute infections. C4b-BP levels were determined by a radioimmunoassay involving the displacement of ¹²⁵I-C4b-BP from an immobilized monoclonal antibody directed against C4b-BP, which has been previously characterized.⁵ To perform the assay, C4b-BP was purified as described¹⁰ and was radiolabeled with ¹²⁵I by the iodogen method¹¹; the ¹²⁵I-C4b-BP had a specific activity of $8 \mu\text{Ci}/\mu\text{g}$. Monoclonal antibody (designated BP45) directed against C4b-BP was bound to Immunobeads (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's directions; approximately $100 \mu\text{g}$ antibody was bound per milliliter of beads. A standard curve of C4b-BP ranging from 0.6 to $0.0375 \mu\text{g/mL}$ was prepared in 20 mmol/L Tris buffer, pH 7.4, containing 150 mmol/L NaCl and 10 mg/mL serum bovine albumin (Sigma Chemical Co, St Louis). To initiate the assay, $50 \mu\text{L}$ of a 1:100 dilution of patient plasma in Tris/saline/albumin buffer, $50 \mu\text{L}$ of the Tris/saline/albumin buffer containing 10,000 cpm of ¹²⁵I-C4b-BP, and $50 \mu\text{L}$ of BP45 immunobeads suspended in 20 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl were pipetted sequentially into a 1.5-mL micro test tube (Bio-Rad Laboratories), mixed on a vortex mixer, and incubated at 25°C for two hours. The beads were then suspended in 1.2 mL 20 mmol/L Tris, pH 7.4, containing 2 mol/L NaCl and vortexed, and the beads were removed from suspension by centrifugation in a Microfuge B (Beckman Laboratories, Palo Alto, CA) for two minutes. Unbound antibody was removed by aspiration and the wash step repeated twice. After the final wash, bound ¹²⁵I-C4b-BP was quantitated in a gamma counter (Nuclear Enterprises, Edinburgh). The C4b-BP content in patient samples was calculated from the standard curve. All samples were run in triplicate. The same-day variation was 8%, and the between-day variation was 11%.

Patients. Twenty-one women with a mean age of 27 years (range between 17 and 49 years) who were taking a single OC formulation for more than 6 months were studied. The formulations

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involved were as follows: 1 mg norethindrone plus 0.05 mg mestranol (seven women), 1 mg norethindrone plus 0.035 mg ethinyl estradiol (three women), 0.5 mg norethindrone plus 0.035 mg ethinyl estradiol (one woman), 1 mg ethynodiol diacetate plus 0.05 mg ethinyl estradiol (three women), 1 mg norethindrone plus 0.05 mg mestranol (one woman), 1 mg norethindrone plus 0.035 mg ethinyl estradiol (four women), triphasic 0.05 to 0.125 mg levonorgestrel plus 0.03 to 0.04 mg ethinyl estradiol (one woman), and 1 mg ethynodiol diacetate plus 0.1 mg mestranol (one woman). Twenty-one women not taking OC (mean age, 33.4 years; range, 22 to 64 years) and 21 men (mean age, 33.8 years; range, 18 to 53 years) were also studied. None of the 63 participants had a personal or familial history of thromboembolic disease.

In addition, the protein S status of six men and two women with severe infections were studied and the results compared with those of sex- and age-matched, healthy controls. Two of the patients had cellulitis, four had bacterial pneumonia, and two had pyelonephritis. All patients had WBC counts of 13,500 or above. All study subjects gave informed consent.

Statistical analysis. Levels of both protein S and C4b-BP in women taking OC were compared with those of men as well as those of women not taking OC by means of Student's *t* test.

RESULTS

Women taking OC had a mean total protein S level of $24.3 \pm 3.6 \mu\text{g/mL}$ (mean $\pm$ SD) with a range of 20.0 $\mu\text{g/mL}$ to 29.6 $\mu\text{g/mL}$. This is significantly lower than the $28.6 \mu\text{g/mL} \pm 3.9 \mu\text{g/mL}$ (range, 21.0 $\mu\text{g/mL}$ to 35.8 $\mu\text{g/mL}$) found in women not taking OC ($P < .005$). Men had significantly higher levels of total protein S ($30.9 \mu\text{g/mL} \pm 3.9 \mu\text{g/mL}$; range, 23.7 $\mu\text{g/mL}$ to 42.0 $\mu\text{g/mL}$) than did the women not taking OC ($P < .01$).

The C4b-BP levels were not significantly different among the three groups examined: $244 \mu\text{g/mL} \pm 98 \mu\text{g/mL}$ (mean $\pm$ 1 SD) for women not taking OC, $253 \mu\text{g/mL} \pm 95 \mu\text{g/mL}$ for men, and $233 \mu\text{g/mL} \pm 105 \mu\text{g/mL}$ for women taking OC.

The observed differences in the total protein S antigen levels and the similar C4b-BP levels found in the three groups suggested that the free protein S levels might be significantly different. This was found to be the case. Women taking OC had $86\% \pm 17\%$ free protein S (mean $\pm$ SD) compared with the free protein S in a normal pooled plasma control, which was significantly lower ($P < .1$) than the levels found in women not taking OC ($90\% \pm 20\%$). The free protein S levels were significantly higher ($P < .005$) in men at $109\% \pm 20\%$ compared with the levels found in women not taking OC.

The thromboembolic complications of OC use are often associated with periods of physiological stress such as the postoperative period¹² and during episodes of trauma and infection.¹³ If C4b-BP levels were elevated during physiological stress (ie, an acute-phase reactant), this could shift the protein S to the complexed form by the law of mass action. This shift in protein S could further compromise the already reduced levels of free protein S found in the OC users and might be a further predisposing factor to thrombosis. The eight patients with acute infection had a mean C4b-BP level of $370 \mu\text{g/mL} \pm 66 \mu\text{g/mL}$, which was significantly higher ($P < .005$) than the mean level of $240 \mu\text{g/mL} \pm 62 \mu\text{g/mL}$ found in eight age- and sex-matched controls. Five of the eight patients had reduced free protein S levels as estimated

by crossed immunoelectrophoresis and mildly reduced total protein S antigen (23.4 $\mu\text{g/mL}$ v 27.8 $\mu\text{g/mL}$ in controls, not significant).

DISCUSSION

The 18% decrease in total protein S associated with OC administration appears to be relatively small. However, since protein S is distributed in plasma in two forms (free protein and complexed with C4b-BP), such a decrease in total protein S may be calculated⁵ to result in a 24% decrease in the free protein S level. Based on this calculation, we believe that particular care should be taken in prescribing OC to women who have decreased protein S levels on a congenital basis and to women who have elevated levels of C4b-BP as a result of a chronic inflammatory process. In the former, the already low protein S levels might be decreased even further by a hormonal effect of OC administration. In the latter case, the elevation of C4b-BP level accompanying the inflammatory process would favor a shift in protein S to the complexed, inactive form. In this situation, a further reduction in total protein S antigen due to the hormonal changes secondary to OC could result in an enhanced reduction in free protein S. To determine the actual contribution of the protein S system to the thrombotic complications of OC use, prospective study will be required.

The 54% increase of the C4b-BP level observed in patients with acute infections suggests that C4b-BP may be an acute phase reactant. This significant increase in C4b-BP levels would significantly affect the distribution of protein S in plasma and would result in a 40% decrease in the free protein S level.⁵ Such a drop in the concentration of free protein S might predispose the patient to thrombosis, especially when other concomitant factors predisposing to thrombosis such as stasis or recent tissue trauma are present.

Finally, the 8% increase in total protein S and the 21% increase in free protein found in men compared with women not taking OC suggests that different normal ranges for these parameters be considered for men and women. This may prove to be of particular value in the definition of the lower limit of the normal range where significant overlap between normal and congenitally protein S-deficient populations may occur.

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Modulation of Endothelial Cell Functions by Different Molecular Species of Interleukin 1

By E. Dejana, F. Breviario, A. Erroi, F. Bussolino, L. Mussoni, M. Gramse, G. Pintucci, B. Casali, C.A. Dinarello, J. Van Damme, and A. Mantovani

Different molecular species of interleukin 1 (IL 1) were examined for the spectrum of responses elicited in human endothelial cells (HEC), including synthesis of prostacyclin (PGI₂), tissue-type procoagulant activity (PCA), platelet activating factor (PAF), and plasminogen activator inhibitor (PA-I). The IL 1 preparations utilized for the present study included a natural, partially purified IL 1, a preparation purified to homogeneity with extensive homology with the derived aminoacid IL 1 β (p17) sequence denominated "22K factor," murine recombinant IL 1 α , human recombinant IL 1 α (p15) and β (p17). Natural, partially purified IL 1, a mixture of α and β species, induced the entire spectrum of

responses in HEC. Production of PA-I was elicited by all forms of IL 1 tested. PGI₂ and PCA were elicited by "22K factor" and by human recombinant IL 1 β and α but not by murine recombinant IL 1 α . PAF synthesis was stimulated by murine and human recombinant IL 1 α but not by human recombinant IL 1 β and 22K factor. Thus the available different molecular forms of IL 1 elicit largely but not completely overlapping patterns of responses in HEC. The IL 1 pathway of regulation of HEC functions might provide a basis for novel strategies in therapeutically oriented research on vessel wall disorders.

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SOLUBLE products released by lymphocytes and macrophages are potent regulators of various functions of vascular cells, such as proliferation, migration, production of colony stimulating factors, and expression of class II histocompatibility (Ia) antigens.¹⁻⁶

Specifically, the activated monocyte product interleukin 1 (IL 1) has been reported to induce multiple responses in human endothelial cells (HEC).⁷ Others and our group have recently shown that this monokine induces prostacyclin (PGI₂)⁸⁻¹⁰ and platelet activating factor (PAF)¹¹ synthesis in endothelial cells (EC) from human umbilical vein. Bevilacqua et al¹² established that IL 1 stimulates expression of a tissue factor-like procoagulant activity (PCA); increase in plasminogen activator inhibitor (PA-I) production by HEC after IL 1 was also observed.¹³

IL 1 is a family of polypeptides, biochemically heterogeneous but with some common biological properties.¹⁴ At least two dissimilar human gene products have been cloned with limited homology, denominated α (p15) or β (p17).¹⁵⁻¹⁷ One murine IL 1 molecular species was also cloned and appeared to be closely related to the corresponding human α IL 1 form, with 62% matching and 93% conserved sequences.^{18,19}

IL 1 has pleiotropic effects, its targets including immunocompetent cells, liver, central nervous system (CNS), synovia, bone, and muscle.²⁰ The multiplicity of the biological activities attributed to IL 1 and the heterogeneity of the components of the IL 1 family raise the question of whether distinct molecules have distinct biological roles. Attempts to relate different molecular species to the diverse effects of IL 1 on a series of tissues and cellular targets have largely failed. Wood and co-workers were able to dissociate the effect of human IL 1 α v IL 1 β on the stimulation of PGE₂ production by porcine synoviocytes. However, intraspecies differences on the activities of the different molecules of IL 1 have not been described.²¹ Here we tested the activity of the series of IL 1 molecules so far available, including recombinant preparations, on HEC. We report that the different molecules elicit a largely but not completely overlapping spectrum of responses on these cells.

MATERIALS AND METHODS

IL 1 preparations. IL 1 preparations utilized for the present study included:

1. A commercially available purified preparation obtained from *Staphylococcus albus*-stimulated human monocytes and purified by affinity chromatography employing immobilized rabbit antibodies and gel filtration (Ultrapure IL 1, Genzyme, Boston)²²; we will refer to this material as human natural IL 1 (hnIL 1).
2. A pure preparation from mitogen-stimulated human mononuclear cells, purified to homogeneity; the N-terminal aminoacid sequence of this material, referred to as "22K factor," has been described²³ and shows extensive homology with the derived aminoacid IL 1 β sequence.
3. Murine recombinant IL 1 (lot 14430-21) from the P388 D₁ cell line¹⁸ was a kind gift of Dr Lomedico (Roche Institute, New York). Since this clone shows extensive homology with the human IL 1 gene designated α ,^{17,19} we will refer to this material as murine recombinant IL 1 α (mrIL 1 α). This preparation had a specific activity of 10⁵

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U/ μ g as evaluated by the costimulatory assay on thymocytes, as described below, and contained 0.23 μ g/U guanidine.

4. Human recombinant IL 1 (p17) expressed in *Escherichia coli* from aminoacid 71-269¹⁵ homologous to a subsequently isolated clone designated β .¹⁶ We will refer to this as human recombinant IL 1 β (hrIL 1 β). The specific activity of this preparations was 2×10^2 U/ μ g.

5. Human recombinant IL 1 α (p15)¹⁶ (hrIL 1 α) was kindly supplied by Dr Gillis (Immunex, Seattle). The specific activity was 10^5 U/ μ g.

IL 1 preparations contained no detectable endotoxin as assessed by the negative Limulus assay on samples containing 100 U/mL of IL 1. IL 1 activity was evaluated in a costimulatory assay with C3H/HeJ thymocytes as responding cells and phytohemagglutinin (0.5 μ g/mL) as stimulus.⁸ A partially purified IL 1 preparation²⁴ was assigned a value of 1,000 U/mL and used as standard in each assay.

Results from triplicate cultures were plotted on a linear regression against the standard preparation and expressed as half maximal U/mL.

Cells. Human umbilical vein endothelial cells were isolated and cultured in medium 199 supplemented with 20% newborn calf serum as previously described in detail.²⁵ The cells were used at the first passage at confluence. After the growth medium was removed the cells (2.5 to 3.5×10^5 cells in a 4 cm² culture well) were washed once with 1 mL phosphate buffer saline (PBS) and incubated at 37 °C with 600 μ L of culture medium in the presence or absence of increasing concentrations of the IL 1 preparations. The time of incubation required to obtain maximal stimulation for each activity was selected on the basis of earlier time-course experiment.^{8,11-13}

Prostacyclin (PGI₂). PGI₂ production was evaluated in the cell supernatant after 24-hour incubation with IL 1. After this period the supernatants were removed from the culture wells, filtered, and stored at -20 °C until tested for 6-keto-PGI₂, the stable PGI₂ metabolite. 6-keto-PGF1 α was measured by a radioimmunoassay (RIA) as previously described in detail.²⁶ Analysis by thin layer chromatography and mass spectrometry²⁵ showed that these cells produce 6-keto-PGF1 α as the main arachidonic acid metabolite.

Platelet activating factor (PAF). PAF was measured in the cell extract after six-hour incubation with IL 1 by biological assay after thin layer chromatography purification, as described previously in detail.¹¹ The product was identified as PAF by its behavior in chromatographic systems, the recovery of biological activity, the physicochemical properties, and susceptibility to lipases.¹¹

Procoagulant activity (PCA). PCA was evaluated in the cell extract after four-hour incubation with IL 1 by a standard one-stage clotting assay (plasma recalcification) as described.²⁷ The standards for the coagulation assay were a 1:10 dilution of rabbit brain thromboplastin. Procoagulant activity in the cell extract was expressed in units of thromboplastin per milligram protein. The type of procoagulant activity was identified using human plasmas selectively deficient in factors II, VII, IX, or X (MERZ-DADE, Duding, Switzerland). Further characterization was performed by the use of known inhibitors of cell procoagulants, namely cysteine protease inhibitors HgCl₂ and iodoacetamide (Sigma Chemical, St. Louis).

Plasminogen activator inhibitor (PA-I). PA-I activity was determined after 24-hour incubation as the capacity of the cell extract to inhibit plasminogen activator-induced plasmin generation in a spectrophotometric assay employing a chromogenic substrate for plasmin as described previously.²⁸

RESULTS

All five IL 1 preparations had IL 1 activity as defined by their ability to stimulate the proliferation of thymocytes in

the presence of phytohemagglutinin (data not shown). The half maximal units of IL 1 for the various preparations were calculated relative to an IL 1 preparation utilized in one of the collaborating laboratories²⁴ (AE and AM). Subsequent comparisons were made on the basis of the concentrations of these agents, which were equally active in the mouse thymocyte costimulatory assay. It has to be mentioned that as evaluated from this test the specific activity of mrIL 1 α was 10^5 U/ μ g protein; of hrIL 1 α , 10^5 U/ μ g; and of hrIL 1 β , 2×10^2 U/ μ g. Thus we had to use 500 times more hrIL 1 β to obtain concentration equally active on the thymocyte costimulatory assay. We have previously reported⁸ that hnIL 1 increased PGI₂ synthesis in vascular cells at concentrations ranging from 1 to 10 U/mL. These observations were now extended to homogenous natural human IL 1 ("22K factor"), hrIL 1 β , and hrIL 1 α (Fig 1). In contrast, mrIL 1 α at concentrations as high as 100 units/mL (corresponding to 1 ng/mL) did not elicit production of PGI₂ in HEC, as shown by the experiment presented in Fig 1.

A similar pattern was observed when PCA was examined (Fig 2). The homogeneous "22K factor," hrIL 1 β , and hrIL 1 α stimulated synthesis of PCA as efficiently as hnIL 1, whereas mrIL 1 α was ineffective at concentrations as high as 100 U/mL.

We have recently observed that hnIL 1 and mrIL 1 α elicited PAF production in HEC.¹¹ As shown in Fig 3, besides hnIL 1 and mrIL 1 α , hrIL 1 α was also an effective inducer of cell-associated PAF in HEC, whereas the "22K factor" had no such activity up to 60 U/mL and hrIL 1 β showed statistically significant activity only at concentrations as high as 100 U/mL (corresponding to 500 ng/mL). HnIL 1, mrIL 1 α , and "22K factor" stimulated production of PA-I in HEC in a comparable way (Fig 4). Since mrIL 1 α failed to induce PGI₂ production and PCA in HEC (see above), it was important to assess whether this preparation contained inhibitors of IL 1-elicited response.

Therefore we incubated HEC for four hours at 37 °C with 50 U/mL of mrIL 1 α mixed with 5 U/mL of 22K factor and measured the PCA produced by these cells. When the cells

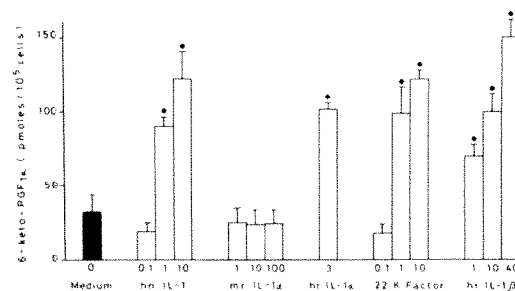


Fig 1. Effect of hnIL 1, mrIL 1 α , hrIL 1 α , "22K factor," and hrIL 1 β on 6-keto-PGF 1 α production by HEC. The numbers below the columns indicate the U/mL of the IL 1 forms, as calculated from the thymocyte costimulatory assay as described in the text. The black column reports the control mean value of 6-keto-PGF1 α produced in the absence of IL 1. The values are means $\pm$ SEM of four separate experiments. In each experiment all the IL 1 molecular species were tested on the same cell culture. * $P < 0.01$ v control values by Dunnett's test.

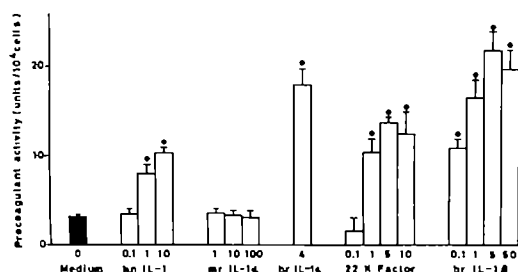


Fig 2. Effect of hnIL 1, mrIL 1 α , hrIL 1 α , "22K factor," and hrIL 1 β on HEC procoagulant activity. The numbers below the columns indicate the U/mL of the IL 1 forms, as calculated from the thymocyte costimulatory assay. The black column reports the control mean value of PCA produced in the absence of IL 1. The values are means $\pm$ SEM of three separate experiments. In each experiment all the IL 1 molecular species were tested on the same cell culture. * P < 0.01 v control values by Dunnett's test.

were stimulated by the "22K factor" alone, the PCA produced was 22.8 ± 3 U/10⁴ cells; mrIL 1 α induced 1.4 ± 0.1 U/10⁴ cells and the combination of the two agents 18.9 ± 3 U/10⁴ cells (the values are means $\pm$ SEM of four replicates, and the results were similar in one additional experiment). It is concluded that the mrIL 1 α preparation does not interfere with "22K factor" activity on PCA production. In addition, since mrIL 1 α contained 0.23 μ g of guanidine per unit, HEC were incubated with up to 12 μ g/mL guanidine alone or in combination with 10 U/mL of hnIL 1; no effects on HEC production of PGI₂, PAF, PCA, or PA-I were observed.

DISCUSSION

The first aim of the present study was to assess whether the modulation of endothelial cell functions by IL 1, which was described by using crude or natural partially purified IL 1, was also effected by purified recombinant molecules. Human natural IL 1 elicited the entire spectrum of HEC responses examined in the present study, thus confirming previous observations.^{8,11-13} This preparation contains the different species, α (p15) and β (p17), of IL 1.²² In contrast, when recombinant IL 1 molecules (or natural material purified to homogeneity in the case of "22K factor") were examined, differential responses were elicited by the dif-

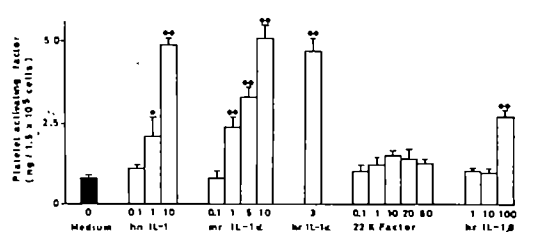


Fig 3. Effect of hnIL 1, mrIL 1 α , hrIL 1 α , "22K factor," and hrIL 1 β on PAF production by HEC. The numbers below the columns indicate the U/mL of the IL 1 forms as calculated from the thymocyte costimulatory assay. The black column reports the control mean value of PAF produced in the absence of IL 1. The values are means $\pm$ SEM of four separate experiments. In each experiment all the IL 1 molecular species were tested on the same cell culture. * P < 0.05; ** P < 0.01 v control values by Dunnett's test.

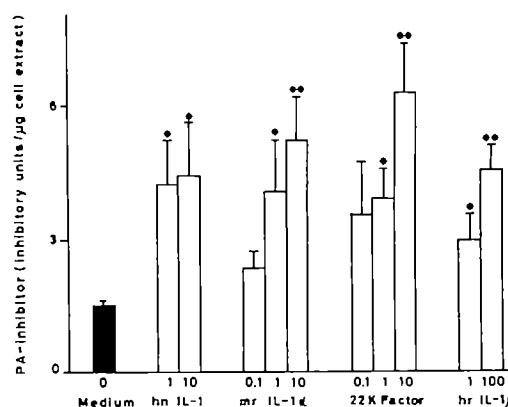


Fig 4. Effect of hnIL 1, mrIL 1 α , "22K factor," and hrIL 1 β on PA-I production by HEC. The number below the columns indicate the U/mL of the IL 1 forms as calculated from the thymocyte costimulatory assay. The black column reports the control mean value of PA-I produced in the absence of IL 1. The values are means $\pm$ SEM of three separate experiments. In each experiment all the IL 1 molecular species were tested on the same cell culture. * P < 0.05; ** P < 0.01 v control values by Dunnett's test.

ferent molecular species examined. All the IL 1s tested induced synthesis of PA-I in HEC. Murine recombinant IL 1 α stimulated the synthesis of PAF in HEC but was consistently inactive in inducing PGI₂ and PCA. Murine recombinant IL 1 α is highly homologous to the human α species.^{17,19} However, in contrast to the murine α species, human recombinant IL 1 α stimulated PCA and PGI₂ synthesis (in the limited number of assays in which we could test this particular IL 1).

We examined the effects on HEC of two preparations of IL 1 β , a natural purified material ("22K factor") and a recombinant preparation. Both IL 1 β preparations elicited the entire spectrum of HEC responses examined with the possible exception of PAF: induction of PAF was only observed with recombinant IL 1 β at a concentration of 100 U/mL, corresponding to 500 ng/mL. Under the same conditions murine recombinant IL 1 α induced PAF at 1 U/mL corresponding to 0.01 ng/mL as previously reported.¹¹ Thus IL 1 β seems to be 100 (on a U/mL basis) or 50,000 (on an ng/mL basis) times less effective than the α species.

The apparently different response of HEC to α and β IL 1 species for PAF production is the first evidence of an intraspecies functional dissociation between these two IL 1 forms in the same cell type. The observation that hnIL 1 stimulates PAF synthesis excludes the possibility of a peculiar effect related to the recombinant nature of the two α species. Conversely the lack of effect of the β forms of IL 1 does not seem to be simply related to their lower activity on HEC, as demonstrated by their effectiveness on the other HEC functions tested. Thus the data presented here indicate that different molecular species of IL 1 elicit largely but not completely overlapping patterns of responses in HEC.

The in vivo response to partially purified IL 1 assessed by fever, neutrophilia, changes in plasma divalent cations, or fibrinogen concentrations, differs in various animal species^{29,30}; furthermore, biochemically distinct IL 1 prepara-

tions can be differently affected by these species-related restrictions. It has not been elucidated whether these differences reflect the presence of target organ-specific inhibitors in the preparations, differences in the *in vivo* bioavailability, or different cell susceptibility. Through the analysis of the activities of four distinct preparations of human IL 1 separated by size or charge, Wood and co-workers²¹ observed no clear-cut differences among them, with the exception that the stimulation of PGE₂ production by pig synoviocytes was only induced by the PI 5.5, 15,000 Kd mol wt IL 1 preparation. These authors considered the possibility of the presence of inhibitors in the inactive preparations. However, the slight inhibitory activity observed in some of the IL 1 preparations was not strong enough to completely account for their apparent inactivity.

In this paper the peculiar spectrum of responses elicited by mrIL 1 α , in respect to the hrIL 1 α , might be related to the murine origin of this clone and suggests that slight differences in the molecular structure may greatly affect functional properties.

In conclusion, the results presented here show that molecules with IL 1 activity have some selectivity in effects on HEC functions and emphasize the potential complexity of the fine regulation of leukocyte-vessel wall interactions. The HEC activities considered in this study, ie, PGI₂, PCA, PAF and PA-I, might be important in the modulation of vascular tone and thrombus formation. In particular, PAF produced by IL 1-stimulated HEC is mostly cell associated¹¹ and appears to be appropriately located to interact with adjacent leukocytes and eventually platelets and to promote their activation. The IL 1 pathway of regulation of these HEC functions could thus provide a basis for novel strategies in therapeutically oriented research on vessel wall disorders.

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CORRESPONDENCE

G6PD IN *PLASMODIUM*-INFECTED ERYTHROCYTES

To the Editor:

Roth et al¹ have recently studied the reduction of oxidized glutathione (GSSG) in lysates prepared from normal and glucose-6-phosphate dehydrogenase (G6PD)-deficient human erythrocytes infected with *Plasmodium falciparum*. They found that the rates of GSSG reduction in infected G6PD-deficient lysates were elevated slightly, in comparison with uninfected cells, but were still substantially lower than rates in normal cells. They therefore concluded that the parasite-derived G6PD could not be considered a substitute for the normal host enzyme activity in alleviating the vulnerability (of host and parasite) to oxidant stress.

This interpretation is not, however, supported by the data, since it is based on the implicit assumption that the *in vivo* rate of GSSG reduction is related directly to the G6PD activity. In fact, the rate of GSSG reduction in normal intact cells is nearly linearly dependent on the activity of hexokinase²; G6PD activity is therefore present in considerable excess with a control coefficient estimated to be 0.006, compared to a value of 0.995 for hexokinase.² Consequently, the rates of GSSG reduction measured by Roth et al¹ (in diluted lysates supplied with NADP⁺ and not glucose but glucose 6-phosphate (G6P) at concentrations sufficient to saturate G6PD) are not an indication of the cells' ability to withstand oxidative stress, but rather are analogous to a coupled enzymic assay for measuring G6PD activity. The effect of bypassing the physiologically rate-limiting hexokinase reaction is demonstrated by comparison of the normal rate of production of reduced glutathione (GSH) found in intact cells (22 $\mu\text{mol/h/g Hb}^2$) with the value of 785 $\mu\text{mol/h/g Hb}$ found by Roth et al, in diluted lysates.

Comparison of the GSSG reduction rate measured in freeze-thaw lysates (supplied with G6P and NADP⁺) of normal and G6PD-deficient cells is therefore misleading, since the rate determined in

normal cells is not attained *in vivo* with the natural substrate, glucose. Sonicate-lysates (supplemented with niacinamide and NADP⁺ and supplied with glucose) would be a better system for studying comparative rates, since these preparations show rates of GSH production identical to those in intact cell suspensions.² Even these comparisons would have to be treated with caution, however, since hemolysis abolishes any heterogeneous distribution of G6PD activity which may be present in suspensions of intact cells.

Indeed, it is possible that sonicate-lysates of infected G6PD Mediterranean cells (supplied with glucose) would show rates of GSH production not much lower than uninfected normal lysates, since the infected G6PD-deficient lysates¹ had GSH production rates (29 to 83 $\mu\text{mol/h/g Hb}$), with G6P as substrate, that were higher than normal cells² supplied with glucose as substrate (22 $\mu\text{mol/h/g Hb}$). If this were the case, however, it would not be sufficient evidence to conclude that the parasite enzyme alleviated the vulnerability of host and parasite to oxidant stress, since physical separation of G6PD (presumably located within the parasite) from GSSG in the erythrocyte cytosol may prevent this. The homogenization caused by hemolysis should therefore be avoided when investigating whether the parasite enzyme can replace the deficient host enzyme in protecting against oxidative stress; the only unequivocal way to approach this problem is therefore to determine the extent of GSH regeneration in intact parasitized G6PD-deficient cells, following oxidation of GSH with, for example, *t*-butyl hydroperoxide (as in ref 2).

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To the Editor:

Thorburn and Kuchel have correctly pointed out some limitations in our recent studies on *P. falciparum*-infected normal and G6PD deficient red cell lysates.¹ However, we find that their interpretation of the data does not change our conclusion concerning the poor capacity of the infected G6PD-deficient red cell to resist oxidative stress. They also have likely overestimated the ability of their computer model,² based entirely on the study of normal uninfected red cells, to deal with the complexities of G6PD deficiency or malaria-infected red cells, especially simultaneously.

It has been known for some time that G6PD and pentose shunt activities in normal, unstressed red cells are well below maximal levels, which can be obtained *in vitro* by adjusting a number of variables in intact red cells as well as in lysates.³ The writers are correct in asserting that our assay of pentose shunt activity overestimates the *in vivo* rate—particularly in unstressed, uninfected red cells. We deliberately framed our study to operate at near-maximal G6PD activity so that at least the upper possible limit of a response

to an oxidative stress could be estimated. We reasoned that if even these maximal activities were not adequate for dealing with oxidative stress, then it would be unlikely that intact cells with lower G6PD activity could perform differently. Thorburn and Kuchel do concede that perhaps G6PD Mediterranean red cells when infected can reduce as much oxidized glutathione (GSSG) as normal, uninfected and unstressed red cells. However, when normal red cells are stressed, they can augment pentose shunt activity at least ten- to fifteenfold.⁴ Merely bringing pentose shunt activity up to the normal, unstressed resting level is not likely to be sufficient for dealing with oxidative stress. Thus, we see no essential conflict between our interpretation of the data and that of Thorburn and Kuchel.

The problem of performing studies on intact cells v lysates (or homogenate/sonicates) is not new in biochemistry. The relatively new possibility of using nuclear magnetic resonance (NMR) for intact cell studies represents an advance in this field and certainly deserves exploration. However, unequivocal results are not likely to be forthcoming, because it will be difficult to tell which compartment is yielding a signal for glutathione or other metabolites.

Furthermore, if the molecular exchange among the compartments is rapid compared with the NMR relaxation times being measured, then an average signal from all compartments may be seen and nothing more. This would not be very different from extracting all the metabolite of interest from a homogenate/lysate of all compartments and measuring it by a more classical technique. The problem of compartmentalization in parasitized red cells alluded to by Thorburn and Kuchel is indeed a difficult one which deserves consideration. How many compartments are there? Should we consider the red cell membrane, red cell cytoplasm, parasitophorous vacuole membrane, the parasitophorous vacuole itself, parasite membrane, parasite cytoplasm, parasite nucleus, and the parasite food vacuole each as separate compartments? Are the Maurer's clefts yet another compartment? Can NMR compartments be distinct from anatomical ones? Such considerations seem to confound bold assertions that there is an "unequivocal way" to approach these problems. Nevertheless, we have also performed intact cell experiments using acetylphenylhydrazine (APH) as the oxidant stress.³ We observed enhanced reduced glutathione stability when infected red cells were challenged with APH and compared to uninfected normal red cells challenged in the same way. When glucose was omitted from the incubation medium, reduced glutathione disappeared more rapidly from infected cells than from uninfected ones. This finding suggests that metabolic communication between the parasite and red cell cytoplasm does occur. The use of *t*-butylhydroperoxide does produce a rapid loss of reduced glutathione, but also kills the parasites (Roth et al, unpublished observation). Thus, in an NMR experiment as suggested by Thorburn and Kuchel, using this reagent, one would be detecting signals from the red cell cytoplasm and a compartment containing the cadaver of a malaria parasite. While this experiment might be interesting, it would hardly be unequivocal. (APH also kills parasites, but more slowly.)

We come next to the computer model of Thorburn and Kuchel which states that hexokinase activity alone controls the activity of the pentose shunt. This model, derived from studies of the red cells of a single normal individual, did not include data from red cells in which G6PD activity approaches zero; nor does it consider an

infected red cell containing a separate parasite hexokinase⁶ (whose kinetic characteristics are unknown) as well as a completely different pathway (based on parasite-derived glutamate dehydrogenase) for the generation of NADPH (and hence GSSG reduction), which is totally independent of hexokinase. In such a complex system, it is unlikely that a linear relationship between human host cell hexokinase activity and GSSG reduction will apply. The concept that the G6PD-dependent pathway of GSSG reduction is much smaller than indicated by studies of dilute lysates also emphasizes the possible importance of the glutamate dehydrogenase pathway which, although small in infected lysates, could account for as much as 50% of the total GSSG reducing capacity in vivo.

Finally, we note the work of Morelli et al⁷ which Thorburn and Kuchel cite in their paper² in support of their interpretation of the pivotal role of hexokinase in controlling the pentose shunt. Morelli et al state: "... in G6PD deficient erythrocytes, unlike in the normal cells, hexokinase no longer plays any rate-limiting role on the glucose utilization through the HMS [hexose monophosphate shunt] which is rather controlled by the low levels of G6PD activity." We are unable to explain why Thorburn and Kuchel cite this reference when it supports their conclusions, but ignore it when it does not.

In summary, we believe that there is no serious conflict in the interpretation of our data, which are a first attempt at characterizing a difficult problem. We thank Thorburn and Kuchel for pointing out limitations and other interpretations of our experiments as well as for suggesting interesting further experiments both with lysates and intact cells. The suggested experiments have much merit although by themselves, they are unlikely to lead to an "unequivocal" interpretation in such a complex system as the parasitized human erythrocyte.

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MIXED CHIMERISM AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM

To the Editor:

Blazar and coauthors¹ describe the use of restriction fragment length polymorphisms (RFLP) of DNA fragments for the investigation of chimerism and origin of leukemic relapse following allogeneic bone marrow transplantation (BMT). Analysis of RFLP provides more complete information about chimerism than cytogenetics, because all nucleated cells are evaluated whether proliferating or

not. Unlike red cell typing, analysis of RFLP allows more insight into chimerism and is independent of transfusions. In our opinion the most important questions are (1) the origin of leukemic relapse from host or donor cells, and (2) the significance of mixed chimerism as a prognostic factor of impending relapse.

However, the data of analysis given by the authors are weak. For example, in patient No. 10, analysis of the recipient's normal and leukemic hemopoiesis was not performed prior to BMT. The conclu-

sion of leukemic relapse in host type cells rests entirely on the occurrence of a single new band during relapse (ref 1, Fig 2).

Fig 3 and the respective legend¹ are confusing: the bands of lanes 3 and 5 are different, but the samples (MNC) are taken from the same day, ie, day 15 after BMT.

Demonstration of mixed chimerism depends on the sensitivity of the RFLP technique, but the sensitivity was tested only for probe pDP 34. The sensitivity may vary with the amount of material available. We have used isoenzymes for studying chimerism and could detect 1% to 2% of 10^5 cells plotted.

In an informative case we could demonstrate leukemic relapse of host type restricted to granulopoiesis together with a minority of mature granulocytes of host type.² In this patient previous analyses

had revealed complete chimerism. In another case we found mixed chimerism persisting for more than a year without leukemic relapse. Therefore, we believe that mixed chimerism after BMT is not necessarily a sign of poor prognosis, nor is complete chimerism a sign of good prognosis regarding leukemic relapse. In support of this hypothesis further data need to be collected.

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To the Editor:

We agree with Drs Mittermüller and Kolb that two of the more important questions relating to leukemia relapse are the donor or host origin of the leukemia relapse and the significance of mixed chimerism in predicting relapse in patients with underlying malignancies. Therefore, we have been prospectively performing DNA analysis on allogeneic transplantation patients in our institution since June 1984. We have now studied 95 donor-recipient pairs pretransplant and have data available on 65 of these patients posttransplant. DNA analysis has permitted us to document definitively the donor or host origin of patients who have experienced leukemia relapse or posttransplant lymphomas¹ and the degree of engraftment of patients who are at higher risk for potential graft failure,^{2,3} as well as to analyze the kinetics of lineage-specific engraftment. The purpose of our report⁴ was to demonstrate the comparative utility of this technique as opposed to speculating on the questions addressed by Drs Mittermüller and Kolb.

Since we are now screening all allogeneic donor and recipient pairs pretransplant for donor-specific and host-specific polymorphisms, we have been able to demonstrate conclusively the donor or host origin of patients undergoing leukemia relapse. This will eliminate the type of retrospective analysis which was conducted in our first series of experiments (ref 4, Fig 2).

As a means of determining graft stability, we have used the prior samples as internal controls for the quantitation of the most recently obtained specimen whenever possible. In Fig 3,⁴ lanes 3 and 5 were generated from the same DNA sample obtained 15 days posttransplant. Lanes 1 through 3 were generated from the same autoradiogram,

while lanes 4 through 7 were generated from a second autoradiogram. The densitometric scanings of the original autoradiograms were remarkably similar, although, as described,⁴ there can be many factors contributing to differences in the accuracy of the final autoradiographic analysis.

The sensitivity of DNA analysis has been consistently reported to be 1% to 5%, regardless of the probe utilized. In our study, we used the probe pDP 34 to determine the sensitivity of this technique. However, similar results have been obtained by two other groups of investigators: one group⁵ used a probe that hybridized to a portion of the immunoglobulin gene; the other group⁶ used probe D14S1. Each of the groups has reported sensitivity of 1% to 5%. In addition, we have compared enzymatic analysis to DNA analysis for patients who have inherited metabolic disorders. In contrast to DNA typing, enzyme typing was more likely to be influenced by post-BMT variables affecting their expression.⁷

We have also observed several patients with persisting Philadelphia chromosome-positive cells who have remained in clinical remission. We concur that mixed chimerism after transplant is not necessarily a sign of poor prognosis but rather indicates that incomplete myeloablation of either normal cell populations or malignant clones probably has occurred. We anticipate that the data generated by these types of studies will provide useful information as to the frequency of this occurrence.

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LACK OF EFFECT OF HEPARIN ON FACTOR XIa

To the Editor:

A report by Beeler et al in the May 1986 issue of *Blood*¹ claims to provide evidence that "plasma concentrations of antithrombin III completely saturated with heparin would become the dominant factor in the inhibition of factor XIa." This statement appears to differ from our summary also published in *Blood*² in October 1982 that "heparin, at concentrations used in clinical practice, does not accelerate the inactivation of human factor XIa by antithrombin III." Therefore, we decided to analyze the possible source of the differences in the two reports.

First, it is important to consider the concentrations of heparin used in both studies. The experiment of Beeler et al, in a purified system, used a single concentration of heparin, 5.88×10^{-6} mol/L, which, based on their specific activity of 350 USP U/mg and a mol wt of 27,000 daltons, yields a concentration of 14.4 USP U/mL. This "saturating" concentration is 40-fold higher than the average pharmacologic concentration of 0.35 USP U/mL found during clinical anticoagulation and fivefold higher than that employed in cardiopulmonary bypass. In our study, we used a range of concentrations from 1 to 30 U/mL in a purified system using both amidolytic and coagulant assays (not esterolytic as stated by Beeler et al). We found no difference in the rate of inactivation of factor XIa by antithrombin III at 1 U/mL heparin in the purified systems. Unlike Beeler et al, we extended our study into plasma systems and were able to confirm the lack of effect of 1 U/mL heparin on the rate of inactivation of factor XIa.

Second, we also examined the suprapharmacologic concentration of heparin used in the study of Beeler et al. We documented a maximal 4-fold increase in the rate of inactivation. In our study, at 23 °C, which assumed the plasma ATIII concentration to be 4.7 μ mol/L, the inactivation rate constant without heparin was 5.4×10^3 mol/L⁻¹min⁻¹ and 2.7×10^4 mol/L⁻¹min⁻¹ at 30 U/mL heparin. When these experiments were performed at 37 °C (unpublished results) using the plasma antithrombin concentration reported by Beeler et al of 2.5 μ mol/L for plasma concentration, the second order rate constants were 3.1×10^4 mol/L⁻¹min⁻¹ without heparin and 1.51×10^5 mol/L⁻¹min⁻¹ with 30 U/mL heparin. Therefore, our data in the presence of heparin is in good agreement with that of Beeler et al, whose calculated second order rate constant is 1.59×10^5 mol/L⁻¹min⁻¹. However their second order rate constant in the absence of heparin of 4.7×10^3 mol/L⁻¹min⁻¹ is only 15% of our value, explaining the apparent 40-fold increase.

Several problems in their experimental procedures may explain their lower value. No controls are presented in their paper for the loss of activity with factor XIa alone, or factor XIa with heparin. Indeed the loss of activity in the absence of heparin is stated to be approximately 50% of the rate constant making its accuracy suspect.

Furthermore, contact proteins demonstrate biphasic loss of activity upon contact with plastic³ making a single time-point correction factor in error. In the experiments performed by Beeler et al, single reactions for each point were used which may lead to greater error in calculations of the actual concentrations of initial activity. When first order kinetics are performed by subsampling from a single reaction vessel, as in our study, one can extrapolate to time zero in order to determine the actual initial concentration of enzyme in the reaction mixture. Moreover, pseudo-first-order reaction rate constants are not accurately determined unless the reaction has undergone at least one half-life, which was not shown in the paper of Beeler et al. Further, our values for the inactivation rate of factor XIa by antithrombin III without heparin in purified systems⁴ agree closely with those observed in plasma. Thus, it appears that the differences observed by Beeler et al stem from an underestimation of the inactivation rate in the absence of heparin.

Our experiments in both purified and plasma systems at pharmacological concentrations of heparin lead us to reassert our conclusion that α_1 -antitrypsin, not antithrombin III, is the major inhibitor of factor XIa in plasma. Our previous study, in collaboration with Dr Rosenberg,⁵ indicates that heparin had minimal effect in accelerating the inactivation of plasma kallikrein by antithrombin III in purified systems and no effect in plasma. A recent study in *Blood* from our laboratory⁶ indicates that heparin had little effect on factor XIIa in purified systems and in plasma. Finally, in a recent study presented at the American Society of Hematology in December 1986 in collaboration with Dr Peter Harpel, we showed evidence for activation of the contact system during clinical cardiopulmonary bypass in the presence of 5 U/mL heparin. Thus, we find that heparin and antithrombin III, although critically important for thrombin, factor Xa, and possibly factor IXa, play little role in the regulation of the contact system of blood coagulation.

McNeely and Griffith⁷ demonstrated that in the presence or absence of 0.5 U/mL of heparin, the amounts of radiolabeled factor IX cleaved in plasma after exposure to glass (contact activation), phospholipid, and calcium were identical. They conclude, in agreement with us, that heparin has no significant effect on the reaction rate between antithrombin III and the enzymes preceding factor IX in the intrinsic pathway.

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To the Editor:

Colman and Scott have provided a detailed critique of our studies. We have previously pointed out numerous experimental problems in their recent investigations of the kinetics of factor XIa-antithrombin-heparin interactions and do not find it necessary to repeat them here.¹ In any case, one of our pictures is more compelling than repeating a myriad of arcane kinetic arguments. Western blot analyses of our reaction mixtures graphically show the generation of large amounts of factor XIa-antithrombin complex in the presence of optimal levels of heparin but no visible interaction product in the absence of the mucopolysaccharide (Figure 2¹). Unpublished data indicate that the factor XIa-antithrombin complex generated in the absence of heparin would have been detected if it were present at as little as 2.5% to 5.0% of the final concentration of factor XIa-antithrombin interaction product formed in the presence of mucopolysaccharide. Thus, heparin must be augmenting the antithrombin-dependent neutralization of factor XIa by 20- to 40-fold. We cannot even claim any great originality for this particular result since Kurachi and Davie² reported a similar finding employing SDS-PAGE and have reached similar conclusions about the magnitude of the mucopolysaccharide-induced augmentation of factor XIa-antithrombin interactions. Based upon the available evidence, we believe that our estimate of the acceleration of factor XIa-antithrombin interactions by heparin (40-fold) is more likely to be correct than that of Scott and Colman (two- to fourfold). Indeed, the original studies of this phenomenon conducted by us more than a decade ago³ appear to be valid.

The effects of heparin therapy on factor XIa-antithrombin interactions were not considered within our publication.¹ However, we did suggest that antithrombin fully complexed with the endogenous

anticoagulant active heparan sulfate of the endothelium could represent a critical biologic mechanism for neutralizing factor XIa within the circulation. We believe this to be the case because of the dramatic acceleration of factor XIa inhibition by antithrombin in the presence of saturating levels of mucopolysaccharide; the unusually strong dependence of the velocity of factor XIa neutralization on selective concentration of antithrombin by mucopolysaccharide on the surface of endothelial cells [see rate equation for factor XIa neutralization¹], and the known relatively slow actions of other protease inhibitors within the blood. This scenario might be quite different during heparin therapy when only a small fraction of antithrombin is complexed by the anticoagulant and when the protease inhibitor might not be concentrated within a small region of the vascular tree. We hope that our results lead other investigators to examine the intriguing possibility that the endogenous heparan sulfate-antithrombin system is critically involved in regulating the in vivo activity of the contact phase of the coagulation mechanism rather than generate endless disputes over the exact extent of inhibition of factor XIa by antithrombin in the presence of heparin.

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DISCRIMINANT ANALYSIS FOR PROBABILITY OF HEMOPHILIA A CARRIERSHIP

To the Editor:

Green et al¹ recently reported a multilaboratory study on hemophilia A carrier testing. They concluded that a linear discriminant function of Factor VIII coagulant activity (F.VIII:C) and von Willebrand factor antigen (VWF:Ag) adjusted for age and ABO blood type provided the best discrimination and that the ratio F.VIII:C/VWF:Ag gave poor results. Our experience has been different. Using a similar analysis, we find the ratio method to be just as accurate and much easier for clinical use.

We have used both a bivariate linear discriminant function, prepared as described by Elston et al,² and a univariate discriminator, the natural logarithm of the ratio F.VIII:C/VWF:Ag, as described elsewhere.³ The ratio has several advantages. The well-documented⁴ effects of age on F.VIII:C and VWF:Ag are approximately equal, and the ratio shows no significant regression on age in

groups of 49 carriers, 81 normal women, and 100 blood donors (unpublished observation). Also, no significant difference in F.VIII:C/VWF:Ag between Type O and non-O women was reported by McCallum et al.⁵ This was confirmed in our blood donor sample. Therefore, neither age nor ABO blood type need be introduced into the discriminant if the F.VIII:C/VWF:Ag ratio is used. Green et al¹ apparently used age-adjusted levels in calculating their ratios.

Table 1 shows our experience with discriminant analysis using the ratio method. Carrier and normal subjects are divided into those used in calculating the discriminant (Group I) and those tested later (Group II). The discrimination is slightly better than that reported by Green et al,¹ although our sample size is considerably smaller. We found fewer misclassifications with the ratio (6.9%) than with the bivariate discriminant produced from the same data (9.2%, data not shown). Average odds favoring carriership were 30:1 in the carriers

Table 1. Use of the Ratio of F.VIII:C/VWF:Ag for Carrier Detection

	Misclassifications	
	No.	%
Carriers		
Group I	3/23	13.0
Group II	3/26	11.5
total carriers	6/49	12.2
Normals		
Group I	2/38	5.3
Group II	1/43	2.3
total normals	3/81	3.7
All subjects	9/130	6.9

and 1:5.9 in the normals; however, we question the accuracy of this method for comparing the discriminants. We too have noted unusual results at the extreme ends of the distributions with both bivariate and univariate discriminants. In particular, subjects with very high F.VIII:C/VWF:Ag may fall in the upper tail of the noncarrier

distribution, where f_n and f_c are both very small numbers, occasionally producing falsely high likelihood ratios. This appears to happen more often with the ratio method. In using the discriminants clinically, these aberrant results are easily recognized. In the data of Green et al,¹ the average odds and misclassification rates for normals using ratios might be lower if such results were removed or modified.

Use of a ratio of F.VIII:C/VWF:Ag to assess probability of carriership is both conceptually and computationally simpler. It produces a low level of misclassification and by avoiding some of the statistical complexity could allow more laboratories to establish their own discriminants. It would be interesting to see whether unadjusted ratios could be used to produce a simple "universal" discriminant.

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To the Editor:

Miller and Aledort suggest that the greater accuracy, in our sample of 336 carriers and 137 normal women,¹ of a universal linear discriminant in comparison to a universal ratio discriminant may be due to the presence of a few individuals with falsely high likelihood ratios. That such is not the case is evident from scatterplots of the odds for carriership for the two discriminants (Figures 1C and 1D¹). In these plots, subjects who are assigned higher odds for carriership by the linear discriminant appear as points below the diagonal line, and vice versa; it is then clear that the linear discriminant assigns higher odds to carriers, and lower odds to normals, than does the ratio, for a substantial majority of subjects. Similar results were seen when nonadjusted ratios were used (data not shown). Odds greater than 100:1 were trimmed to 100:1 in these plots, as well as in computing the average odds for carriership (Table 5¹).

We feel that this graphic method for comparing the two discriminants is superior to the misclassification rate used by Miller and Aledort, since the genetic counselor is interested in the odds rather than a classification per se, and since the misclassification rate may be unduly influenced by the behavior of the discriminant for individuals having odds near 1:1 (for whom the discriminant is of little use). In any case, in our study the linear discriminant also had the lower misclassification rate (Table 5¹). Miller and Aledort used a linear discriminant prepared as described by Elston et al,² which does not include a correction for ABO blood type. This correction should be included, since it improves discrimination for some subjects.¹

The method outlined in the appendix to our paper reduces calculation of the odds for carriership to a few simple arithmetic manipulations of the data, which can easily be programmed on a programmable hand calculator. No statistical software is required. Ease of use is thus essentially the same for the two discriminants, and should have no bearing on a laboratory's choice between them. We know of at least two laboratories not participating in the original study that have implemented our method, with satisfactory results.

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GRANULATION IN ALL BLASTS

To the Editor:

I read with interest the detailed report of Fradera and colleagues¹ that discussed the diagnostic confusion caused by the presence of variably sized azurophilic granules in the cytoplasm of blasts of acute lymphoblastic leukemia (ALL) and described the cytochemical and ultrastructural characteristics of these inclusions. Contrary to the authors' impression, the occurrence of such granules in ALL is neither uncommon nor unreported.²⁻⁴ Moreover, several ultrastructurally different organelles can produce inclusions mimicking the azurophilic granules of early myeloid cells at the light microscopic level, and different granules have different cytochemical properties. Anomalous azurophilic granules occurring in blasts and immature cells of lymphoid leukemias can be roughly divided as follows:

Crystalline: Elongated crystals that can either represent immunoprotein, usually in an envelope of rough endoplasmic reticulum, or nonimmunoglobulin structures with a regular periodicity (often 55 Å), have been reported in chronic lymphocytic leukemia⁵ and prolymphocytic leukemia.⁶ The inclusions containing ribosomal material are pyroninophilic, whereas other crystalline inclusions contain acid hydrolases and phosphatases suggesting lysosomal origin. Crystals rich in immunoglobulin often may fail to react with Romanowsky stains, producing a clear, rectangular to rhomboidal space in the cytoplasm of the blast or immature lymphoid cell.^{7,8}

Ribosomal Variants: Malignant lymphoid cells of both chronic⁹ and acute¹⁰ lymphocytic leukemias have been reported to contain variably sized azurophilic inclusions that ultrastructurally resemble the lamellar-ribosomal complex of the hairy cell. Anday⁹ further

reports that granules resembling the lamellar-ribosomal complex possess pyroninophilia abolished by ribonuclease pretreatment.

Lysosomal/mitochondrial: Several authors describe azurophilic granules in blasts of lymphoid origin that have been proven by electron microscopy (EM) to originate from lysosomes, mitochondria, or combinations of both organelles.^{11,12} The cytochemical reactions of such granules vary, but most often they are similar to those seen in the cells of the patient of Fradera and colleagues,¹ containing nonspecific acetate or butyrate esterase—usually fluoride resistant—acid phosphatase, and glucuronidase. Many also react to period acid-Schiff (PAS).^{4,11} Rarely, granules of this sort contain lipid and react to Sudan Black B, creating major diagnostic confusion.¹³ They do not react with granulocytic specific lysosomal stains such as myeloperoxidase and ASD chloroacetate esterase.

The appearance of such anomalous azurophilic granules in ALL blasts is not unusual, as they were found in ~10% of the new ALL patients studied at St Jude's.⁴ Hematologists and pathologists should not diagnose French-American-British (FAB) M1 acute leukemia on the morphological appearance alone, especially in a clinical context more suggestive of ALL. Cytochemical confirmation of the granulocytic origin of the leukemic cells is essential. Given the occasional report of sudanophilic granules in ALL blasts,^{4,13} the stains used should be granulocyte enzyme-specific, such as myeloperoxidase or ASD chloroacetate esterase. Only such testing can avoid the diagnostic problem so well illustrated by Fradera and colleagues.¹

RICHARD A. SAVAGE

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To the Editor:

We read with interest the comments by Dr Richard Savage and would like to clarify one point and perhaps raise others.

When we decided to study and publish our case with the abnormal granules¹ we were convinced, and still are, that we were reporting a most unusual finding. Unfortunately, Dr Savage missed the point of

our report. We did not report a case of ALL with azurophilic granulation. Indeed, we were most impressed by the fact that the granules were actually not azurophilic. This was clearly described in the light microscopy section of our report (p 407) and can be observed in the color photomicrographs accompanying the article. The granules were described as dark purple, and we believe that the data as presented illustrated the fact that they appeared more like

inclusions than granules, as is generally observed in hematopoietic cells.

In addition, Dr Savage errs when he states that the cytochemical findings in our case were similar to those reported by others^{2,3} who studied azurophilic granules in ALL blasts. Although we agree that the cytochemical evaluations of the blasts containing the azurophilic granules reported by others are similar, our findings were not the same. In our case, the granules gave negative results with all stains tested. Dr Savage's confusion probably arises from the fact that we also described the cytochemical evaluation of the lymphoblasts of our case. These studies revealed an unusual reaction obtained in the lymphoblasts of our patient with the α -naphthyl acetate stain illustrated in Fig 2 of our paper. These studies, however, should not be confused with those of the abnormal granules that were the main subject of our publication.

Dr Savage's letter raises other interesting points in regard to azurophilic granules. We were surprised to read his statement: "The presence of such anomalous azurophilic granules in ALL blasts is not unusual, as they are found in ~10% of the new ALL patients studied at St Jude's Hospital." This information was based on a report by Stein and colleagues² describing a detailed evaluation of five cases of granulated ALL in which the authors screened 66 cases of ALL for the presence of prominent azurophilic intracytoplasmic granules. In our experience with 140 adult patients with ALL in the last 10 years, this has been an extremely rare finding in adults and as such was mentioned in the now classic paper by Bennett and co-workers⁵ in which the French-American-British (FAB) classification was described in 1976 and which included both pediatric and adult patients. Furthermore, in an even more detailed definition of the morphological criteria important in the classification of ALL by

Bennett and co-workers⁵ in 1979, the presence of azurophilic granules was not mentioned. Nevertheless, if the more common presence of these azurophilic granules, as reported by Stein and co-workers² could be corroborated by other pediatric centers specialized in the study of leukemia, this could lead to a revision of the now accepted FAB classification with the recognition of granular ALL as a subtype of ALL, type L₂. This also raises the possibility that if azurophilic granulation in ALL blasts has indeed increased during recent years, a larger number of cases should be available to allow a better characterization of such granules, including utilization of cytogenetic and electron microscopic studies to determine if they represent a finding of clinical relevance in a homogeneous group of patients.

In summary, Dr Savage's observations have raised new perspectives in the study of morphological findings that may be occurring in growing numbers of pediatric patients with ALL in whom azurophilic granules are apparently more common, and corroborate that large, nonazurophilic granulation in lymphoblasts, such as the ones described by us, are indeed a very rare and unique finding. Our case probably represents one of the very few cases, if not the only well-documented case, of such an entity.

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ERRATUM

In the article entitled "Absence of Tubular Structures and Immunolabeling for von Willebrand Factor in the Platelet α -Granules From Porcine von Willebrand Disease" by Cramer et al, which appeared in the September issue (Volume 68, Number 3, pages 774-778), the diameters of the granule-associated tubules and of the microtubules should be read in Angströms and not in nanometers (200 Å and 250 Å, respectively).

ANNOUNCEMENTS

HOUSTON SOCIETY OF CLINICAL PATHOLOGISTS

Houston, TX
April 25, 1987

Slide seminar on lymphomas and leukemias presented by Robert Collins, MD, and John Bennett, MD.

For further information, contact: James J. Butler, MD, Department of Pathology, Box 85, M.D. Anderson Hospital, Houston, TX 77030.

AMERICAN SOCIETY OF HEMATOLOGY

Spring Symposium on Hematopoietic Growth Factors

Durham, North Carolina
April 20-21, 1987

The American Society of Hematology is holding a two-day symposium on hematopoietic growth factors sponsored by Burroughs Wellcome Company, to be held at the Searle Center in Durham, North Carolina on April 20 and 21, 1987. The topics will include the molecular biology of the hematopoietic growth factors, their biological actions, and the results of in vivo studies with these molecules in experimental animals and humans. Among the participants will be Steven Clark, Malcolm Moore, David Golde, Judith Gasson, Ken Kaushansky, Gordon Wong, Peter Quesenberry, John Adamson, and Colin Sieff.

Additional information and details of the program are available through Dr Wendell F. Rosse, PO Box 3934, Duke University Medical Center, Durham, NC 27710. Individuals interested in submitting abstracts or poster presentations, or preregistering are encouraged to do so by March 1, 1987. Maximum registration: 200.

GEORGETOWN UNIVERSITY MEDICAL CENTER, WASHINGTON, DC "BONE MARROW TRANSPLANTATION: PROGRESS AND PROBLEMS"

Washington, DC
March 28, 1987

For further information contact: Office of Continuing Medical Education, Georgetown University Medical Center, 3800 Reservoir Rd, NW, Washington, DC 20007. Telephone (202) 625-2306.

SHORT COURSE IN MEDICAL & EXPERIMENTAL MAMMALIAN GENETICS

July 20-31, 1986
Bar Harbor, Maine

Conducted by The Jackson Laboratory and Johns Hopkins University School of Medicine. Lectures covering the entire field are supplemented by methodologic workshops in cytogenetics, biochemical screening, linkage analysis, and molecular genetics, as well as demonstrations of mouse mutants and problems in clinical genetics. Held at The Jackson Laboratory. *Inquiries and applications should be made to:* Victor A. McKusick, MD, 292 Carnegie Bldg, Johns Hopkins Hospital, 600 N Wolfe St, Baltimore, MD 21205; or to Thomas H. Roderick, PhD, c/o Training & Education Office, The Jackson Laboratory, Bar Harbor, ME 04609.

**LEUKEMIA SOCIETY OF AMERICA
THIRD ANNUAL NATIONAL SYMPOSIUM**

**Town and Country Hotel, San Diego, CA
March 18-21, 1987**

Designed to focus on the latest advances and techniques in research and treatment of leukemias and lymphomas, the program will address current information and rapidly developing new approaches and programs in studies of the disease. Emphasis will be upon the application of basic science and its relation to clinical practice. The meeting is geared to physicians, nurses, allied professionals, fellows-in-training, residents, and students with major interest and involvement in leukemia and related disorders. The course is accredited for CME by the AACME. It meets the criteria for 17 credits in Category I towards the Physician's Recognition of the AMA. Registered nurses will qualify for 20.7 contact hours of continuing education through the LSA's provider accreditation for continuing education for registered nurses.

There will be a keynote address on "Chromosomes, Genes, and Leukemia" by Peter C. Nowell, MD; Stohlman Memorial Scholar papers; posters; and five minisymposia, dealing with: biological characteristics of clinical trial designs; molecular genetics; application of molecular probes; antimyeloid monoclonal antibodies; and cellular response to growth factors. There will also be daily group breakfasts and an awards banquet. Registration fee: physicians: \$100; nurses, fellows-in-training, residents, and students: \$60. Fees are due by March 11, 1987.

For registration and additional information, contact: LSA Medical Conference, c/o Bostrom Corporation, 435 N Michigan Ave, Suite 1717, Chicago, IL 60611. Telephone: (312) 644-0828.

Grune & Stratton Journals

AMERICAN JOURNAL OF CARDIAC IMAGING	Quarterly
AMERICAN JOURNAL OF KIDNEY DISEASES	Monthly
ARCHIVES OF PSYCHIATRIC NURSING	Bimonthly
BLOOD	Monthly
COMPREHENSIVE PSYCHIATRY	Bimonthly
INFORMATICS IN PATHOLOGY	Quarterly
JOURNAL OF CARDIOTHORACIC ANESTHESIA	Bimonthly
JOURNAL OF CLINICAL ONCOLOGY	Monthly
JOURNAL OF CRITICAL CARE	Quarterly
JOURNAL OF PEDIATRIC NURSING	Bimonthly
JOURNAL OF PEDIATRIC SURGERY	Monthly
JOURNAL OF POST ANESTHESIA NURSING	Quarterly
METABOLISM—CLINICAL AND EXPERIMENTAL	Monthly
PROGRESS IN CARDIOVASCULAR DISEASES	Bimonthly
SEMINARS IN ANESTHESIA	Quarterly
SEMINARS IN ARTHRITIS AND RHEUMATISM	Quarterly
SEMINARS IN DERMATOLOGY	Quarterly
SEMINARS IN DIAGNOSTIC PATHOLOGY	Quarterly
SEMINARS IN HEMATOLOGY	Quarterly
SEMINARS IN NEPHROLOGY	Quarterly
SEMINARS IN NUCLEAR MEDICINE	Quarterly
SEMINARS IN ONCOLOGY	Quarterly
SEMINARS IN ONCOLOGY NURSING	Quarterly
SEMINARS IN OPHTHALMOLOGY	Quarterly
SEMINARS IN ORTHOPAEDICS	Quarterly
SEMINARS IN PERINATOLOGY	Quarterly
SEMINARS IN RESPIRATORY INFECTIONS	Quarterly
SEMINARS IN ROENTGENOLOGY	Quarterly
SEMINARS IN ULTRASOUND, CT, AND MR	Quarterly
SEMINARS IN UROLOGY	Quarterly
SEMINARS IN VETERINARY MEDICINE AND SURGERY (SMALL ANIMAL)	Quarterly
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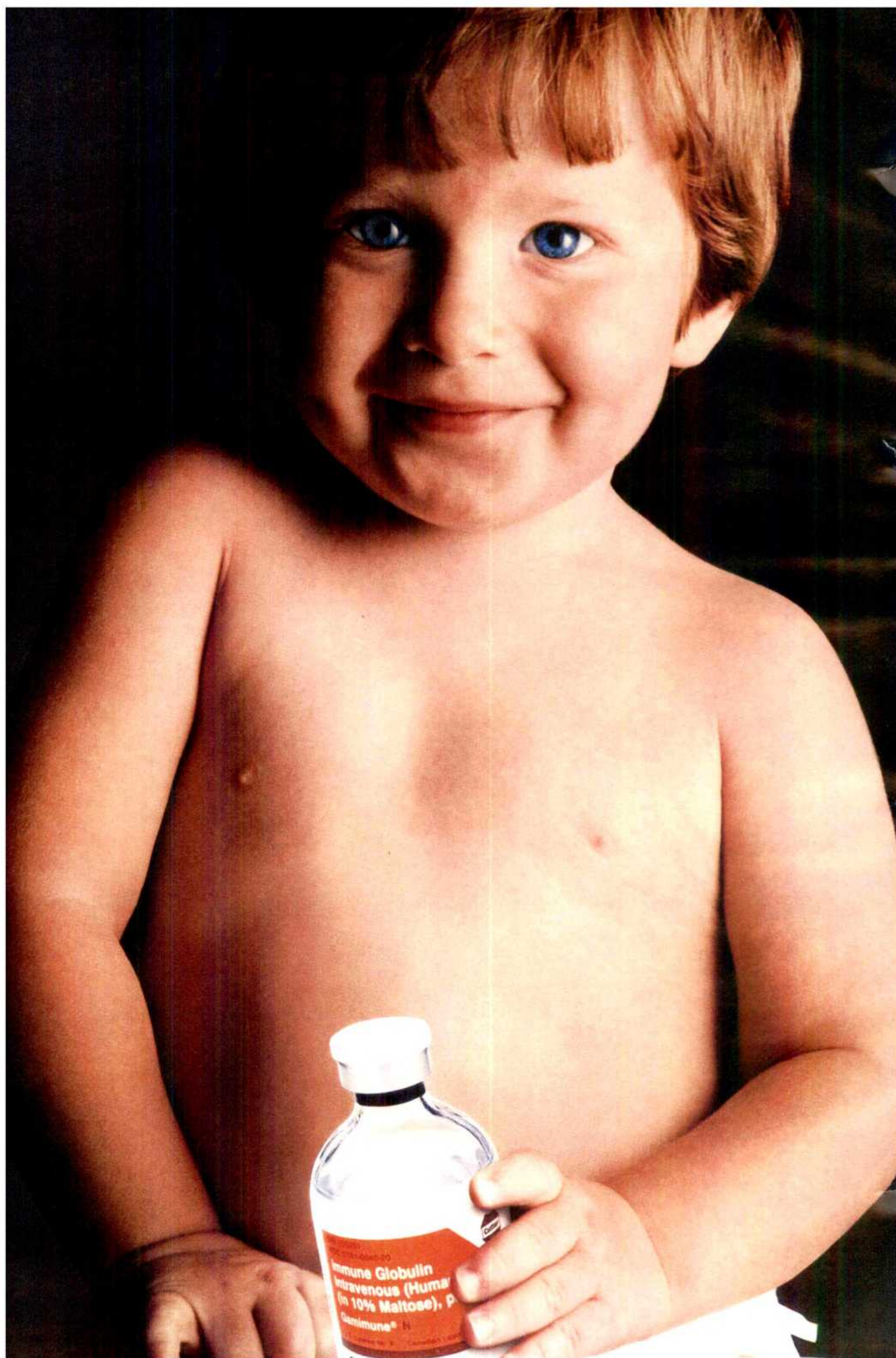
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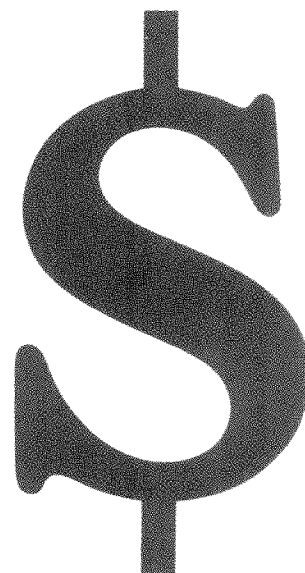
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*The Cohn-Oncley process used to produce Gamimune®N has been challenged *in vitro* with a virus spike of 1×10^5 TCID₅₀ (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune®N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 TCID₅₀ (Tissue Culture Infectious Doses) reduction of HTLV-III.²

Please see following page for brief summary of prescribing information.

GAMIMUNE[®] N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune[®] N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune[®] N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The *in vivo* half-life of Gamimune[®] N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune[®] N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune[®] N at a dose of 150 mg/kg body weight, following a dose of 400 mg/kg body weight in 37 patients; there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune[®] N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune[®] N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune[®] N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune[®] N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune[®] N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune[®] N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune[®] N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune[®] N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune[®] N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune[®] N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune[®] N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PLA¹ alloimmune antibody with platelet anti PLA¹ specificity) also responded to treatment with Gamimune[®] N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N should be considered, in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune[®] N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune[®] N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune[®] N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune[®] N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune[®] N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune[®] N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune[®] N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune[®] N. It is not known whether Gamimune[®] N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune[®] N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune[®] N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune[®] N. Symptoms reported with Gamimune[®] N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N at the infusion site was reported in some cases.

In further studies of Gamimune[®] N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune[®] N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune[®] N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune[®] N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune[®] N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune[®] N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune[®] N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune[®] N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

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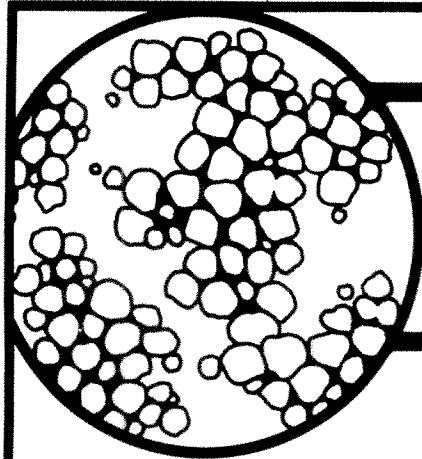
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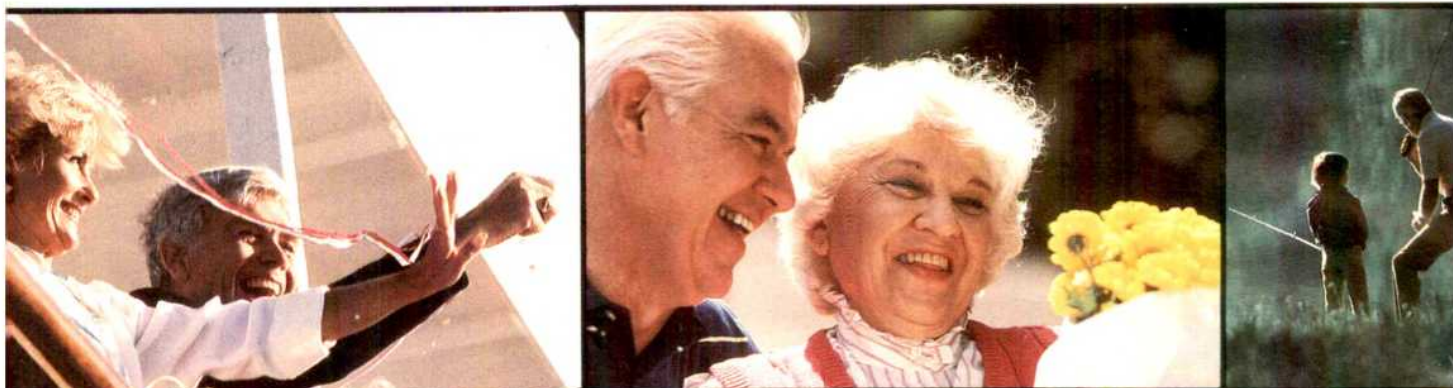
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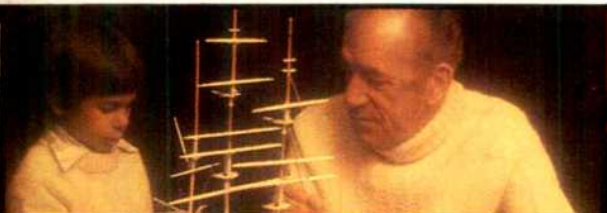
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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B₁₂. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below $5 \times 10^{-8} M$.^{1,2}

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e., trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.^{3,4,5}

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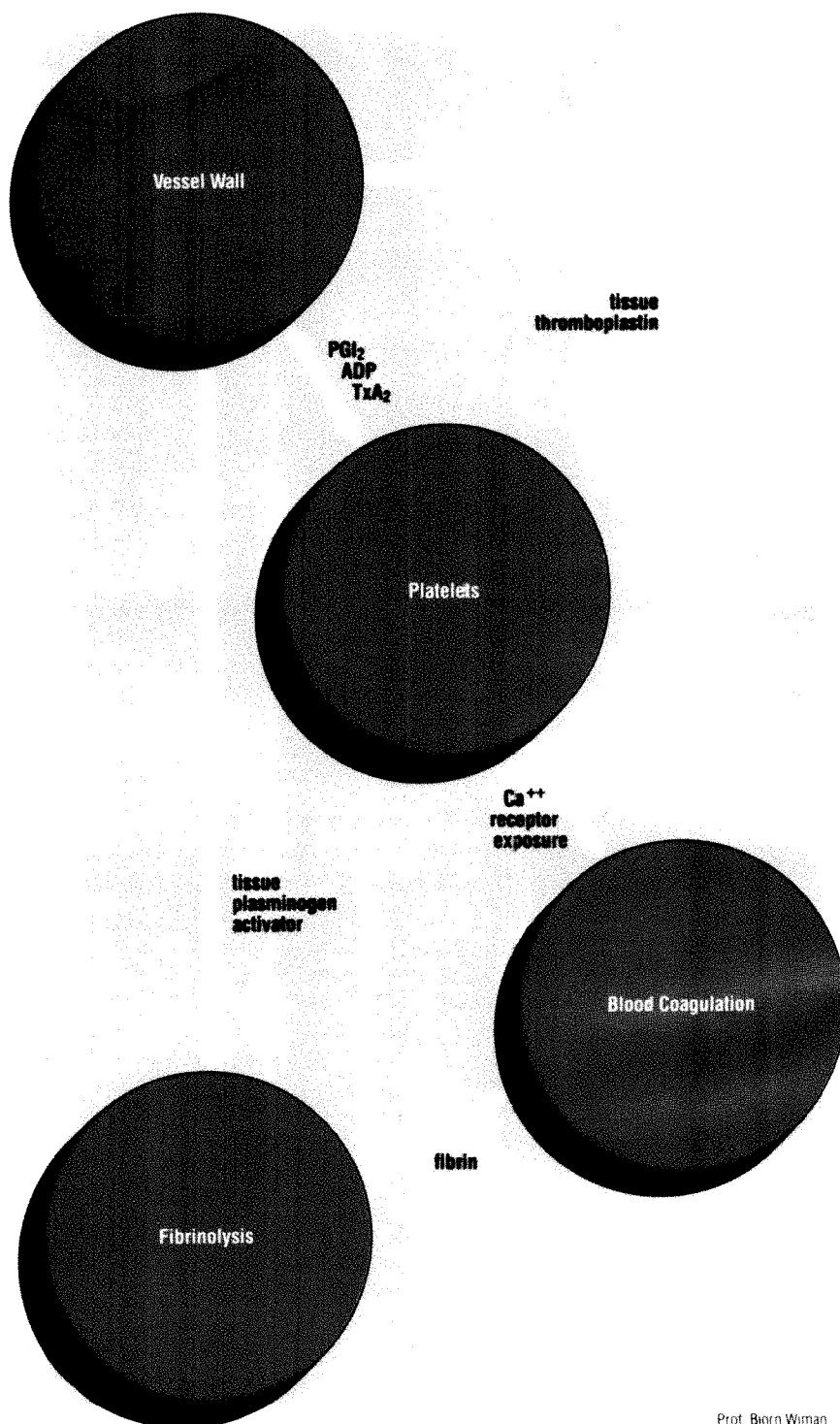
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Radiogenic mucositis or herpes simplex?

See answer under flap.



Early intervention can prevent or reduce HSV complications

A high percentage of immunocompromised patients are at risk for developing HSV infections. Timely therapy with an antiviral agent can avoid the incidence of secondary complications, such as visceral dissemination, and prolonged hospital stay.

Appearances may be deceiving

Both slides are herpes simplex. In immunocompromised patients, mucositis and stomatitis that appear to result from radiation therapy or immunosuppression may actually be caused by herpes simplex. Differential diagnosis can be problematic, but appropriate laboratory tests can aid in the determination.

There is an effective therapeutic agent available

ZOVIRAX I.V. stops viral replication, speeds healing, and reduces pain. It is effective antiviral therapy for treatment of herpes simplex viral infections.

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(acyclovir sodium)
STERILE POWDER / I.V. INFUSION

When the problem begins with a virus,
end it with an antiviral.

The diagnostic
challenge in
immunocompromised
patients...



Please see following page for brief summary of prescribing information.

ZOVIRAX® I.V. (acyclovir sodium) STERILE POWDER / I.V. INFUSION

ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M² every 8 hours (750 mg/M²/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.^{1,2,3}

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.⁴

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.⁵

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.⁶

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M²) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used

in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INfections in IMMUNOCOMPROMISED PATIENTS — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M² at a constant rate over 1 hour, every 8 hours (750 mg/M²/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M ²)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, *et al.*, *Lancet* 1(8235): 1389-1392, Jun. 27, 1981. 2. Wade, *et al.*, *Ann. Intern. Med.* 96(3): 265-269, Mar. 1982. 3. I.D. Meyers, *et al.*, *Am. J. Med.* 73(1A): 229-235, Jul. 20, 1982. 4. Data on file, Burroughs Wellcome Co. 5. A. Mindel, *et al.*, *Lancet* 1(8274): 697-700, Mar. 27, 1982. 6. Z.M. Naib, *et al.*, *Cancer Res.* 33:1452-1463, 1973.



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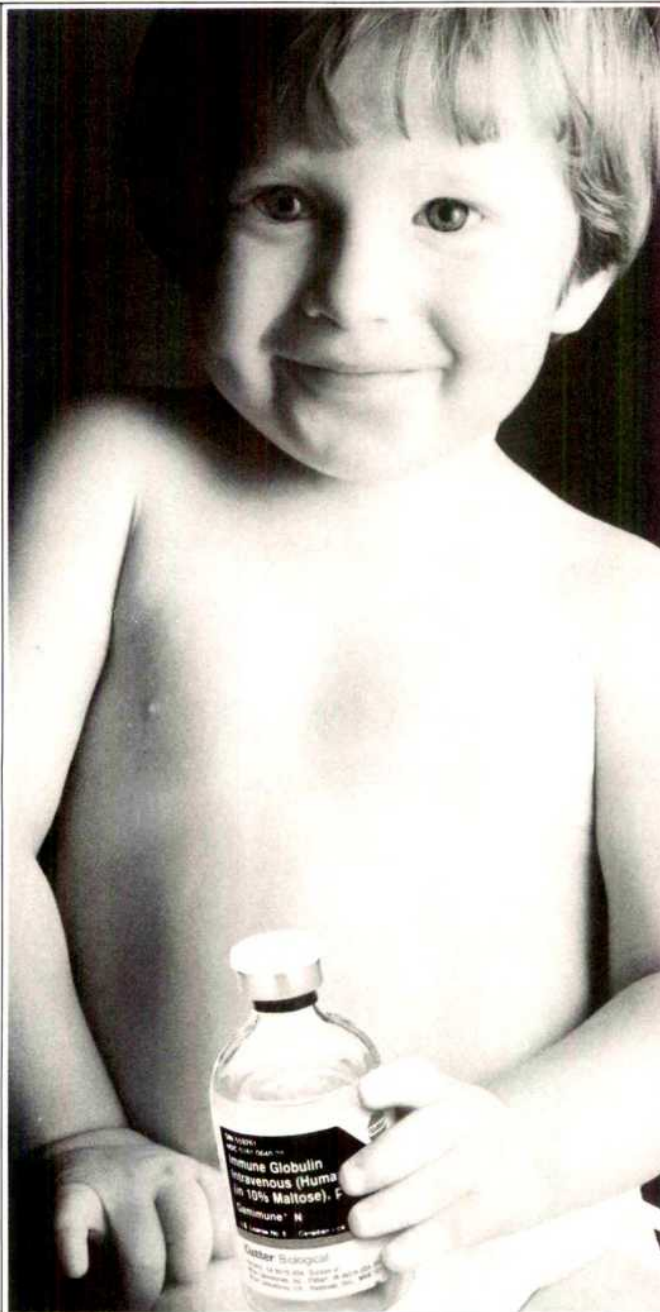
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Cutter's fractionation method (Cohn-Oncley 6 & 9) contains all the necessary steps to produce high purity immune globulin. GAMIMUNE® N contains no foreign contaminants, no detectable levels of protein aggregates or vasoactive enzymes.² With GAMIMUNE® N you get what you expect... IGV containing at least 98% pure gamma globulin.¹

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Intravenous (Human) 5%
(in 10% Maltose), pH 4.25

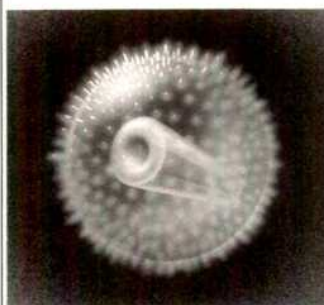


Documented in vitro inactivation of HTLV-III virus[‡]

[‡]The Cohn-Oncley process used to produce Gamimune®N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune®N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 TCID (Tissue Culture Infectious Doses) reduction of HTLV-III.³

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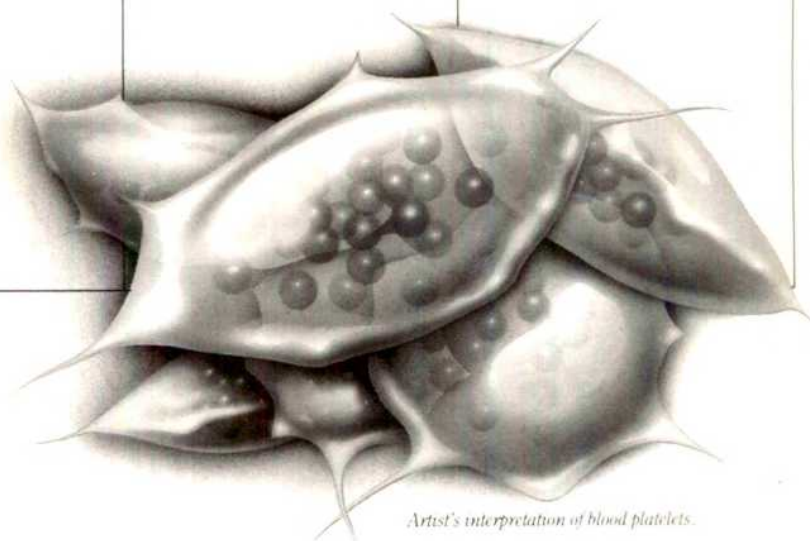
GAMIMUNE®N is manufactured without chemical modification. This means GAMIMUNE®N antibodies maintain normal opsonization capacity, have a normal distribution of IgG subgroups and provide the immunologic activity of normal plasma.¹



Artist's interpretation of HTLV-III virus.

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Dose for dose, GAMIMUNE®N saves money by eliminating valuable reconstitution time in the pharmacy. Gram for gram of protein, GAMIMUNE®N is affordable; there is no premium price for TODAY'S IGIV.



Artist's interpretation of blood platelets.

*It is not possible to predict which patients with ITP will respond to therapy.

¹In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

GAMIMUNE[®] N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune[®] N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune[®] N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The *in vivo* half-life of Gamimune[®] N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3.8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune[®] N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune[®] N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune[®] N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune[®] N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune[®] N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune[®] N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune[®] N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune[®] N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune[®] N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune[®] N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune[®] N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune[®] N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune[®] N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PLA¹ alloimmune antibody with platelet anti PLA¹ specificity) also responded to treatment with Gamimune[®] N at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune[®] N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune[®] N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune[®] N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune[®] N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune[®] N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune[®] N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune[®] N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune[®] N. It is not known whether Gamimune[®] N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune[®] N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune[®] N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune[®] N. Symptoms reported with Gamimune[®] N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune[®] N at the infusion site was reported in some cases.

In further studies of Gamimune[®] N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune[®] N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune[®] N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune[®] N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgias, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune[®] N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune[®] N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune[®] N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune[®] N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune[®] N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune[®] N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes, if well tolerated, the rate may be gradually increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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REFERENCES: 1. GAMIMUNE[®] N Product License Application. 2. Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(Jul-Aug): S382-S390. 3. Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26(4): 394-397.

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*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

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Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. 2. Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. 3. Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. 4. Data on file. Miles Pharmaceuticals.



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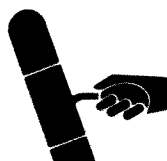
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in their early to midfetal period (days 22 to 70 of gestation). A late-fetal period study (days 79 to 100 of gestation) is in progress and as yet there have been no reports of any increased rate of abortion.

Usage in Pregnancy: Safe use in human pregnancy has not been established. Therefore, Roferon-A should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus. Information from primate studies showed dose-related menstrual irregularities and an increased incidence of spontaneous abortions. Decreases in serum estradiol and progesterone concentrations have been reported in women treated with human leukocyte interferon.⁴ Therefore, fertile women should not receive Roferon-A unless they are using effective contraception during the therapy period.

Male fertility and teratologic evaluations have yielded no significant adverse effects to date.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from Roferon-A, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use: Safety and effectiveness in children under 18 years of age have not been established.

ADVERSE REACTIONS: The following data on adverse reactions are based on the subcutaneous or intramuscular administration of Roferon-A as a single agent for the treatment of 63 patients with hairy cell leukemia during investigational trials in the United States.

Flu-like syndromes consisting of fatigue (89%), fever (98%), chills (64%), myalgias (73%) and headache (71%) occurred in the majority of patients and tended to diminish with continuing therapy. Other side effects such as anorexia (46%), nausea (32%), emesis (10%), diarrhea (29%), dizziness (21%), rash (18%), change in taste (13%), dryness or inflammation of the oropharynx (16%), dry skin or pruritus (13%) and weight loss (14%) were observed with moderate frequency. Less commonly, diaphoresis (8%), paresthesias (6%), numbness (6%), partial alopecia (8%), reactivation of herpes labialis (8%), transient impotence (6%) and arthralgias (5%) were also observed. Rarely (<3%), central nervous system effects including decreased mental status, depression, visual disturbances, sleep disturbances and nervousness, as well as cardiac adverse events, including hypertension, chest pain, arrhythmias and palpitations, were reported. Adverse experiences which occurred rarely and may have been related to underlying disease, included epistaxis, bleeding gums, ecchymosis and petechiae. Miscellaneous adverse events, such as night sweats, urticaria, conjunctivitis and inflammation at the site of injection, were also rarely observed. Roferon-A has also been evaluated for the treatment of many other types of cancer under investigational trials in the United States. These studies generally utilized higher doses (12 to 50 million IU/m²). All of the previously described adverse reactions which occurred in patients with hairy cell leukemia were also observed in patients receiving higher doses. The incidence of most adverse reactions was similar between the two groups, but tended to be more severe in patients who received higher doses of Roferon-A. Some additional adverse effects which occurred in these patients included confusion (10%), hypotension (6%), lethargy (3%) and edema (3%). Adverse experiences occurring in less than 1% of these patients and observed only in patients with malignancies other than hairy cell leukemia are as follows: **Gastrointestinal**—abdominal fullness, hypermotility and hepatitis; **Central Nervous System**—gait disturbance, poor coordination, hallucinations, syncope, seizures, encephalopathy, psychomotor retardation, coma, stroke, transient ischemic attacks, aphasia, aphonia, dysarthria, dysphasia, forgetfulness, amnesia, sedation, apathy, anxiety, emotional lability, irritability, hyperactivity, involuntary movements, claustrophobia and loss of libido; **Peripheral Nervous System**—muscle contractions; **Cardiovascular**—congestive heart failure, pulmonary edema, myocardial infarction, Raynaud's phenomenon and hot flashes; **Pulmonary**—bronchospasm and tachypnea; **Miscellaneous**—excessive salivation and flushing of skin. **Abnormal Laboratory Test Values:** The percentage of patients with hairy cell leukemia or with other types of malignancies who experienced a significant abnormal laboratory test value at least once during their treatment is shown in the following table:

ABNORMAL LABORATORY TEST VALUES

	Hairy Cell Leukemia (n = 63)	Overall Safety Summary* (n = 1019)
Hematologic		
Leukopenia	59%	69%
Neutropenia	39%	58%
Thrombocytopenia	42%	42%
Decreased Hemoglobin	36%	6.3%
Decreased Hematocrit	43%	12.5%
Mean Time to Nadir		
WBC	38 days	22 days
Platelets	19 days	17 days
Hepatic		
SGOT	47%	78%
Alkaline Phosphatase	18%	48%
LDH	12%	47%
Bilirubin	1.6%	31%
Renal/Urinary		
BUN	2%	10%
Serum Creatinine	3%	10%
Uric Acid†	6%	15%
Proteinuria‡	10%	25%
Other Chemistry Tests		
Hypocalcemia	10%	51%
Elevated Serum Glucose‡	33%	39%
Elevated Serum Phosphorus	2%	17%

*Patients with hairy cell leukemia are included in this overall summary.

†The majority of abnormal uric acid values and proteinuria were of minimal severity. Since these abnormalities are commonly observed in patients with advanced malignancy, it is difficult to ascertain their relationship to Roferon-A treatment.

‡Random glucose determination.

Neutralizing antibodies to Roferon-A were detected in approximately 27% of all patients (3.4% for patients with hairy cell leukemia). No clinical sequelae of their presence have been documented thus far. Antibodies to human leukocyte interferon may occur spontaneously in certain clinical conditions (cancer, systemic

ROFERON®-A (Interferon alfa-2a, recombinant/Roche)

lupus erythematosus, herpes zoster) in patients who have never received exogenous interferon.⁵

DOSAGE AND ADMINISTRATION: Hairy Cell Leukemia—The induction dose of Roferon-A is 3 million IU daily for 16 to 24 weeks, administered as a subcutaneous or intramuscular injection. Subcutaneous administration is particularly advisable for, but not limited to, thrombocytopenic patients (platelet count < 50,000) or for patients at risk for bleeding. The recommended maintenance dose is 3 million IU, three times per week. Dosage reduction by one-half or withholding of individual doses may be needed when severe adverse reactions occur. The use of doses higher than 3 million IU is not recommended. Patients should be treated for approximately six months before the physician determines whether to continue therapy in patients who respond or discontinue therapy in patients who did not respond. Patients with hairy cell leukemia have been treated for up to 20 consecutive months. The optimal duration of treatment for this disease has not been determined.

If severe reactions occur, dosage should be modified (50% reduction) or therapy should be temporarily discontinued until the adverse reactions abate. The need for dosage reduction should take into account the effects of prior X-ray therapy or chemotherapy that may have compromised bone marrow reserve. The minimum effective dose of Roferon-A has not been established.

The subcutaneous or intramuscular routes of administration should be used. Subcutaneous administration is particularly suggested for, but not limited to, patients who are thrombocytopenic (platelet count < 50,000) or who are at risk for bleeding.

Injectable Solution:

3 million IU Roferon-A per vial—Each 1 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride, 5 mg Albumin (Human) and 3 mg phenol as a preservative.

18 million IU Roferon-A per vial—Each 1 mL contains 6 million IU of Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride, 5 mg Albumin (Human) and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche.

Sterile Powder for Injection:

18 million IU Roferon-A per vial—Reconstitute with 3 mL diluent and swirl gently to dissolve. When reconstituted with Diluent for Roferon-A, each 1 mL of reconstituted solution contains 6 million IU Interferon alfa-2a, recombinant/Roche, 9 mg sodium chloride, 5 mg Albumin (Human) and 3 mg phenol as a preservative. Each 0.5 mL contains 3 million IU of Interferon alfa-2a, recombinant/Roche. Once the powder is reconstituted, it must be used within thirty (30) days.

The sterile powder and its accompanying diluent, the reconstituted solution and the injectable solution should be stored in the refrigerator at 36° to 46°F (2° to 8°C). Do not freeze or shake.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

HOW SUPPLIED:

Roferon-A Injectable Solution: Vials containing 3 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/mL). Boxes of 10 (NDC 0004-1987-01).

Roferon-A Injectable Solution: Vials containing 18 million IU Interferon alfa-2a, recombinant/Roche (3 million IU/0.5 mL). Boxes of 1 (NDC 0004-1988-09).

Roferon-A Sterile Powder for Injection: Vials containing 18 million IU Interferon alfa-2a, recombinant/Roche, with accompanying diluent. Boxes of 1 (NDC 0004-1993-09).

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1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. *Br J Haematol* 38:161, 1978

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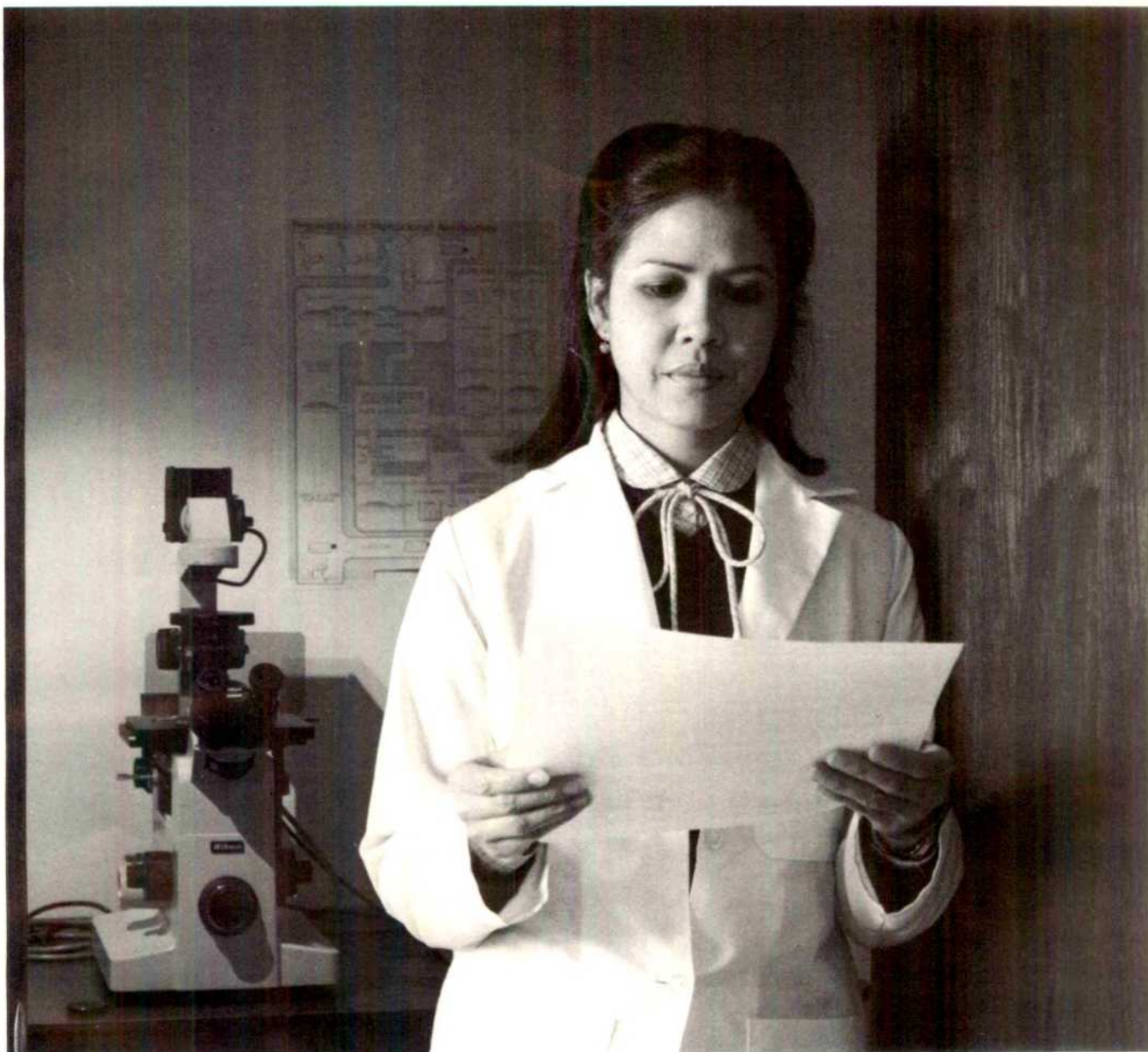
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Immune Globulin Intravenous (Human) GAMMAGARD®

DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8 ± 0.4 .

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.¹ Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.²

As a class, IgG survives longer *in vivo* than other serum proteins.^{2,3} Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.^{2,3,4} The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.^{2,3,4,5}

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has

been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.^{4,5}

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than $10 \mu\text{g/mL}$) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.^{5,6} Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by

monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

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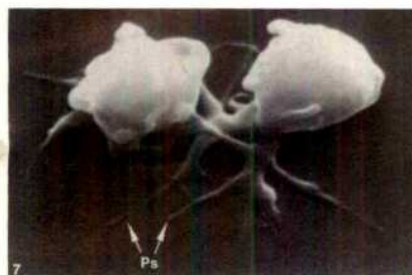
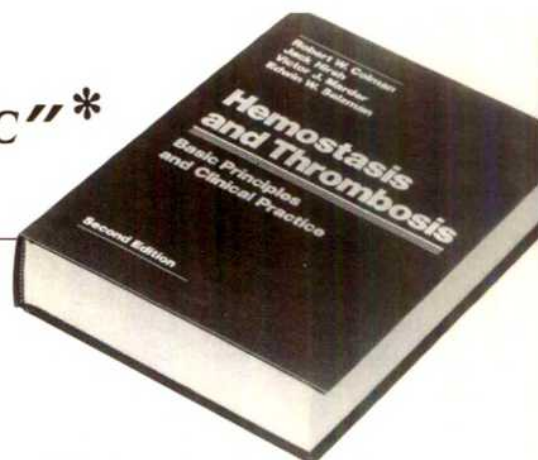
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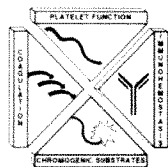
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1987 Helena Hemostasis Advanced Wet Workshops

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A series of comprehensive, advanced, one day Hemostasis Wet Workshops presented by Helena Laboratories. The session will include lectures on the PFI (Proenzyme Functional Inhibitor) Index, platelet aggregation and chromogenic substrates as well as hands-on training with AT-III. Workshop participants will perform the COATEST® Antithrombin Chromogenic Assay using Microtiter plates. This procedure is just one demonstration of cost reduction with advanced technology for your lab.

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When and Where?

1987 Advanced Hemostasis Spring Workshop Schedule

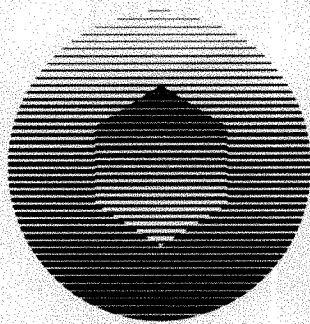
April	6	Raleigh, NC	May	4	Seattle, WA
	7	Miami, FL		5	Portland, OR
	8	Atlanta, GA		6	San Francisco, CA
	9	Memphis, TN		7	Los Angeles, CA
	10	New Orleans, LA		8	San Diego, CA
April	27	Denver, CO	June	1	Boston, MA
	28	Phoenix, AZ		2	Hartford, CT
	29	Dallas, TX		3	New York, NY
	30	San Antonio, TX		4	Philadelphia, PA
May	1	Houston, TX		5	Washington, DC
May	18	Minneapolis, MN			
	19	Chicago, IL			
	20	Detroit, MI			
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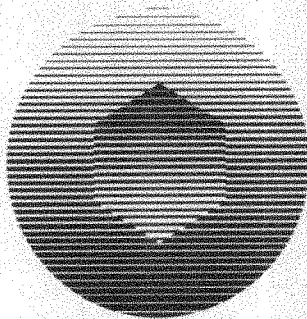
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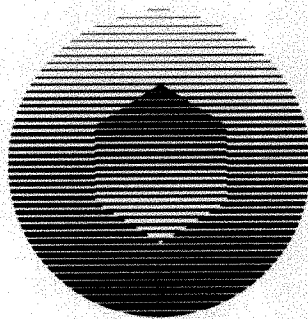


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REVIEW**Protein Kinase C and the Activation of the Human Neutrophil NADPH-Oxidase**

By Alfred I. Tauber

THE NEUTROPHIL leads a short but dynamic life. Attuned to the vagaries of surveillance and destruction, it has developed a complex functional repertoire: Chemotaxis, adherence/aggregation, phagocytosis, degranulation, and oxidative burst require multi-component modes of response which, although highly integrated, may also be studied in dissected form.¹⁻⁶ The problem of signal transduction is a major focus of current study in cell biology, and the neutrophil has become a model of such inquiry, as attention to its various activities has discerned the outlines of its informational response pathways. Within this context, I wish to review the current understanding of the activation mechanism of the respiratory burst enzyme, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, as a paradigm of neutrophil secretory systems. This activity, defective in chronic granulomatous disease (CGD), is the principal defense against a wide variety of bacteria, and thus serves a crucial role in normal host defense.^{3,7} That a normal respiratory burst is dependent on appropriate receptor function, intact activating pathways, and an integral NADPH-oxidase (which in itself is multicomponent),⁷⁻¹¹ predicts multiple sites at which a biochemical lesion may arrest the complex cascade. With recent studies in broken cell systems, important insights into the sequence of activation steps required for normal neutrophil response have been made. By tracing exogenous agonist-induced stimulation of the NADPH-oxidase, the basic elements of the activation sequence are now discerned.

The human neutrophil has several well-defined receptors, including those for various components of C3,¹² immunoglobulin,¹³ the formyl-methionyl (FMLP) bacterial peptides,¹⁴ and leukotriene B₄.¹⁵ A recently described receptor is the ubiquitous protein kinase C (PK-C), a calcium, phospholipid-dependent kinase, activated by exogenous phorbol esters (eg, phorbol myristate acetate, PMA), and endogenously by diacylglycerol liberated by the action of phospholipase or phosphodiesterase.¹⁶ The evidence that receptor-ligand binding is critical in the activation of the metabolic burst is the correlation between ligand binding and biochemical expression. A receptor-binding linkage to NADPH-oxidase activation for PMA has been established.¹⁷⁻¹⁹ Patients with classically described CGD have not been reported to have abnormal receptor affinities as inferred by normal chemotactic and phagocytic responses^{3,8} or phorbol ester receptor binding,¹⁸ but certain patients have demonstrated selected responses to various stimuli and thus exhibit

a defect in the activation apparatus²⁰⁻²²; those with either congenital²¹ or acquired defects³ in PMA-induced oxidase activation are of particular interest. Thus, CGD, which includes all defects of the oxidative metabolic effector system, may be divided into two types: abnormal activating mechanisms and abnormalities of NADPH-oxidase.³

The activation of the human neutrophil by PMA leads to a spectrum of responses, including aggregation,²³ stimulation of the respiratory burst,^{17-19,24-26} depolarization of the plasma membrane,²⁷ enhanced phospholipid turnover,²⁸⁻³⁰ and degranulation of specific granules.³¹ The same constituent of the NADPH-oxidase found in the plasma membrane have been putatively reported in the specific granule membrane; upon PMA stimulation, a subset of these granules is translocated to the plasma membrane, the site of the active respiratory burst enzyme.^{32,33} Of the various stimulus-response-coupled functions defined for the normal neutrophil, I wish to focus on those biochemical phenomena related to the PMA receptor linked to the expression of NADPH-oxidase. In bypassing many associated complex metabolic activities, the PK-C system is a promising avenue for elucidating oxidase activation, at a site just proximal to the final expression of the respiratory burst enzyme. This PK-C dependent pathway is not the sole cascade for oxidase activation, however, since other soluble agonists and phagocytosed particles may bypass PK-C altogether.^{34,35} Although FMLP bypasses PK-C in elicited degranulation and oxidase activation, the chemotactic response is reduced by PK-C inhibition.³⁶⁻³⁸ Definition of parallel and cross-over points in the complex hierarchy of the activation process have yet to be discerned, and there is only general acknowledgment that more than one activation pathway may generate an active oxidase.

Recent studies have focused on the specificity of PMA for PK-C in eliciting a given biologic response. The synthetic diacylglycerol, 1-oleoyl-2-acetylglycerol (OAG), directly

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stimulates PK-C and mimics PMA stimulation of oxidative burst activation.³⁹ Although both PMA⁴⁰ and OAG⁴¹ at submaximal concentrations exhibit synergism with the calcium ionophore, A23187, OAG has an apparent calcium dependence that PMA lacks.⁴² Another difference in response is the enhancement with calcium and arachidonate products observed with OAG stimulation, but not found with PMA.^{43,44} Dramatic functional differences have been observed in the HL-60 differentiation model, in which OAG totally fails to mimic the PMA-induced maturation of this tumor cell line⁴⁵; this apparent dissociation of PMA and PK-C has been confirmed with Bryostatin, another activator of PK-C that blocks PMA-induced HL-60 differentiation.⁴⁶ Interpretation of these data are difficult, however, because another target in addition to PK-C may be involved in this system, modulating the PK-C effect. Bryostatin, however, like OAG, does induce human neutrophil-specific granule release and oxidative burst similar to that of PMA stimulation.⁴⁰ Phosphorylation products were also similar when PMA and Bryostatin were compared in the same system (see below); thus, the ambiguity of the role of PK-C in HL-60 is not evident in the neutrophil.⁴⁷ Furthermore, the evidence for the role of PK-C in PMA-stimulated neutrophils rests most firmly on the reconstitution studies discussed (*sic infra*).

PHORBOL ESTER RECEPTOR

Correlation of phorbol ester's affinity characteristics to activation of the burst established that PMA and its analogue, phorbol 12,13 dibutyrate (PDBu), stimulated NADPH-oxidase through a specific receptor.^{17,18} Extension of studies with intact cells to the subcellular assessment of the oxidase demonstrated a similar dose-response activation profile.¹⁸ This evidence, linking ligand-receptor binding to initiation of the respiratory burst, is important support that NADPH-oxidase is indeed the principal enzymatic activity of the human neutrophil oxidative metabolic response. Characterization of the phorbol ester receptor showed that it was not susceptible to sulfhydryl reactive reagents, stilbene disulfonic acids, or neuraminidase, which explains the earlier findings of a lack of effect of these agents on the generation of O_2^- in PMA-stimulated cells, in contrast to cells stimulated with opsonized zymosan (OZ).^{48,49} The stimulus specificity demonstrated that the inhibition acted on distinct plasma membrane receptors and not on an oxidase exposed to the extracellular medium.

Studies of the PMA receptor suggested new approaches to defining the unknown pathways leading from receptor binding to activation of the oxidase, with the demonstration that the phorbol ester receptor is the calcium/phospholipid-dependent PK-C.¹⁶ In the neutrophil, PK-C exists as a cytosolic aporeceptor, with high phospholipid affinity for phosphatidylserine (PS).^{50,51} Phorbol esters mimic the elaboration of endogenous diacylglycerol required for an active PK-C complex^{16,52} (Fig 1). Diacylglycerol is generated as part of the complex phosphatidylinositol (PI) catabolism, which leads to the elaboration of inositol triphosphate, important for the release of intracellular calcium, a second messenger in diverse stimulus-coupled reactions.⁵³ Normally, diacylglycerol is almost absent from membranes but is

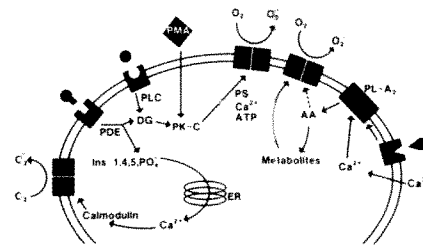


Fig 1. Schema of human neutrophil NADPH-oxidase activation as discussed in text. PDE, phosphodiesterase; DG, diacylglycerol; PLC, phospholipase C; PLA₂, phospholipase A₂; AA, arachidonic acid; PS, phosphatidylserine; Ins 1,4,5-PO₄, inositol triphosphate; O₂⁻, superoxide; ER, endoplasmic reticulum. The NADPH-oxidase is depicted as two adjoining square boxes.

transiently produced from PI in a signal-dependent manner by certain surface membrane receptors.^{54,55} The role of PI turnover and the accumulation of diacylglycerol has been intensively studied in several tissues, and a similar pathway has been described in neutrophils.⁵⁶⁻⁵⁹ PK-C activated in this manner shows multifunctional catalytic activity and appears to play roles in transmembrane control of protein phosphorylation in a variety of mammalian tissues. Phorbol esters are able to substitute for the diacylglycerol, and with PS and Ca²⁺, PMA directly activates the kinase and probably bypasses cyclic AMP- and cyclic GMP-modulated pathways.^{16,52,60} Thus, it is expected that pertussis toxin, which blocks the dissociation of regulatory G proteins and thereby inhibits their role in signal transduction,⁶¹ has no effect on PMA-stimulated cells, in contrast to neutrophils activated with FMLP.⁶² A caution is raised by the observation that a second undefined-G-protein-sensitive site may regulate exocytosis in PMA-activated cells,⁶³ suggesting the complexity of neutrophil response to this single agonist.

In the presence of optimal phospholipid and phorbol esters, the kinase is fully active at 10⁻⁷ mol/L of Ca²⁺, implying that the enzyme may be activated without a net increase in intracellular Ca²⁺ concentration when the appropriate receptors are stimulated. Indeed, PK-C has been reported as active at vanishingly low Ca²⁺ concentrations,^{64,65} and in PMA-stimulated neutrophils, a rise in intracellular calcium is not observed with the fluorescent probe Quin-2.⁶⁶⁻⁶⁸ This observation is based on the use of chemical indicator probes that may introduce an observational artifact.⁶⁹ Stimulation of PK-C only with agents (phorbol esters, teleocidin) other than diacylglycerol demonstrates this low Ca²⁺ dependence. Pontremoli and colleagues recently suggested⁷⁰ that PMA indeed mobilizes intracellular Ca²⁺ but, because of concomitant activation of a Ca²⁺ extrusion pump,⁷¹ a net change in intracellular Ca²⁺ is not observed. The role of Ca²⁺ mobilization in PMA-stimulated neutrophils has also been suggested by the inhibition of O₂⁻ generation observed with agents that inhibit intracellular Ca²⁺ mobilization⁷² or calmodulin,^{73,74} a Ca²⁺-dependent regulator of diverse function. These studies have been difficult to evaluate because of the nonspecificity of the inhibitors used^{67,75}; conclusions as to the role of ancillary Ca²⁺-dependent activities on PMA stimulation of the burst cannot as yet be made. Finally, Dougherty and Nidell showed that agents which alter cytosolic Ca²⁺ levels modulate binding of

phorbol diesters, suggesting a mechanism for the observed synergism between subthreshold activation of Ca^{2+} and PK-C pathways.⁷⁶ In this regard, the low level of recovery from broken cells of NADPH-oxidase with the reconstituted PK-C system (see below) may be due to omission of other metabolic tributaries contributing to PMA-initiated burst activation.

In the case of micromolar concentrations of calcium (a physiologic rise in Ca^{2+} induced by many agonists), the cytosolic form of PK-C and a Ca^{2+} requiring thiol proteinase, calpain, becomes associated with the neutrophil membrane.⁷⁷ The activation of calpain converts PK-C to a proteolytically modified Ca^{2+} /phospholipid-independent form, both in broken cell preparations and in intact cells stimulated with PMA.⁷⁸ Once the translocated PK-C has undergone proteolytic cleavage to a 65-kd species (from 85 kd), it is released to the cytosol in an active form, presumably accessible to other protein substrates. Whether oxidase activation requires membrane-bound PK-C^{51,70} remains to be demonstrated in the reconstituted system. The data thus far suggest that PK-C may be activated by two separate systems with different substrate targets, adding a second level of complexity to the control modulation of C-kinase-mediated functions. Furthermore, the stability of PK-C binding to the membrane appears to vary with the stimulus used.⁷⁹

Physiologic control mechanisms have been complicated further by the recent findings of Cox and co-workers, who demonstrated selective activation of the oxidase with synthetic diacylglycerols.⁸⁰ Of a series of synthetic *sn* 1,2-diacylglycerols, didecanoylglycerol (diC_{10}) induced lysosomal enzyme secretion but failed to stimulate the respiratory burst. Unlike other diacylglycerols that competed for all phorbol ester binding, diC_{10} competed for only 45% of the binding sites. The data showed a correlation of selective PK-C binding with a limited cellular response, and further suggested that a discrete pool of PK-C was responsible for activation of the NADPH-oxidase. These findings are of particular interest in regard to the recent identification of multiple, distinct forms of PK-C by cloning techniques.⁸¹⁻⁸³ The specificity and regulation of response has thus acquired a new molecular complexity.⁷⁸⁻⁸⁰ Determination of type-specific PK-C isoenzymes, with possibly differing activation characteristics and target proteins, promises to add a sophisticated dimension to the study of PK-C. Finally, an endogenous PK-C inhibitory factor (non-protease, non-phosphatase) associated with the specific granules has been partially characterized.⁸⁴ Its role, the substrates of the calpain system, the putative selective target pools of PK-C, and the subcellular localization of activated PK-C require extensive coordinated study to define physiologic control mechanisms of phorbol ester stimulation.

The rapid flux in lipids, mobilization of arachidonic acid (AA) by the activation of phospholipases, and the extensive turnover of membrane phospholipids during phagocytosis represent associated metabolic activities of the respiratory burst.^{3,53,57} Inhibitors of phospholipid turnover do inhibit chemotaxis and phagocytosis and may interfere in the activation sequence of the respiratory burst,^{2,3,5} but there is no direct evidence linking phospholipid turnover to activation of

the burst by PMA, in contrast to particulate stimulation.^{35,56} Phospholipid metabolism accompanies the activation process and likely contributes to membrane fluidity changes seen in the stimulated cell.^{3,85,86} Changes in total phospholipid composition are both time and stimulus dependent, the most dramatic change in PMA-stimulated cells being a net elaboration of PS noted during the first 3 minutes of stimulation.^{29,30} That the PMA aporeceptor is cytosolic and has a uniquely high affinity for PS, which appears most effective for the activated receptor,^{16,51,54} suggests that the PS elaborated has a functional role for PK-C activity, but the mechanism by which PS is elaborated in PMA-stimulated cells is not known.

OZ, which may activate the PI cascade described above,⁵⁶ stimulates O_2^- release by a mechanism distinct from that of PMA.³⁵ This has been amply demonstrated with neutrophils from patients with paroxysmal nocturnal hemoglobinuria³ and a congenital neutrophil dysfunctional case,²¹ in which response to OZ was intact, whereas PMA could not initiate a normal respiratory burst. Only recently have some of the biochemical parameters of the two agonists been defined (Fig 1). PMA differs from OZ stimulation in its susceptibility to a PK-C inhibitor (isoquinolinesulfonamide,⁸⁷) and the lack of PMA-initiated release of radiolabeled AA ($[^3\text{H}]\text{AA}$) from prelabeled cells.³⁵ The specific involvement of phospholipase A2 (PLA_2) in the release of AA in OZ-stimulated cells has been implicated, and the partial reduction of O_2^- formation observed with inhibitors of AA metabolism (BW755C and indomethacin) further supports a linkage between AA release and O_2^- generation in OZ-stimulated cells.³⁵ Finally, like O_2^- generation, $[^3\text{H}]\text{AA}$ release in OZ-stimulated cells is calcium dependent,³⁵ another difference from calcium-independent PMA activation.⁶⁶⁻⁶⁸ It is of interest that exogenous AA stimulates the respiratory burst in intact cells seemingly by the same PLA_2 -dependent pathway as with OZ, but in this case, inhibition of AA metabolism has no effect on O_2^- generation.⁸⁸ In toto, opsonized particle stimulation of the NADPH-oxidase differs from PMA in that the particulate stimulus is PLA_2 mediated, calcium dependent and independent of PK-C. These studies suggest that this physiologic stimulus, binding to plasma membrane receptors, induces an activation cascade biochemically distinct from that generated when PK-C is directly stimulated by phorbol esters. PMA, in bypassing these associated lipid metabolic activities, allows examination of the activation cascade at a point distal to them. This is a useful juncture at which to study the activation of the NADPH-oxidase directly.

*A role for Ca^{2+} in PMA-activated neutrophils has been implicated in preliminary studies, which show that when cells are depleted of intracellular Ca^{2+} , or when intracellular Ca^{2+} mobilization is blocked, O_2^- generation and depolarization are abolished in a Ca^{2+} -free buffer. The effective extracellular Ca^{2+} concentration that restores 50% activity is 100 $\mu\text{mol/L}$ for depolarization and 1 $\mu\text{mol/L}$ for O_2^- production, suggesting different requirements for these functions. Considering the Ca^{2+} requirement of PK-C, these findings are expected, and thus the apparent Ca^{2+} -independence of PMA stimulation is only relatively low as compared to other agonists.^{65a}

PK-C ACTIVATION OF NADPH-OXIDASE

The respiratory burst enzyme, NADPH-oxidase, is maximally stimulated by PMA⁴⁹; the electrons utilized to produce reactive oxygen species arise from the activation of the hexose monophosphate (HMP) shunt, which not only supplies NADPH (the reducing agent) but also stoichiometrically generates one proton for each electron required to reduce molecular oxygen to superoxide.⁸⁹ The protons are secreted largely by a sodium-proton antiporter, whose control of cellular pH appears to regulate cell activation in an as yet undefined manner.⁹⁰ In the initial studies of PK-C activation of the NADPH-oxidase, distribution of PDBu binding was measured.⁹¹ Almost 95% of the specific [³H]PDBu binding was found in the cytosolic fraction of unstimulated, nitrogen-cavitated neutrophils, with a specific binding activity of 8.6 pmol/mg protein. The K_d for [³H]PDBu binding to the cytosolic fraction reconstituted with PS determined by Scatchard analysis was 1.2 nmol/L ($n = 3$). This value is similar to that determined for the partially purified receptors from mouse brain cytosol.⁹² This affinity is considerably higher than that observed in intact cells (38 nm),¹⁸ presumably reflecting the reconstitution of the receptor in PS rather than the actual phospholipids of the neutrophil membranes.

No significant difference was found in the distribution of the binding activity between the subcellular fractions of control and PMA-stimulated neutrophils, in contrast to findings in other studies.^{19,51} Because phorbol ester treatment has been reported to induce changes in apparent PK-C distribution in neutrophils and some other systems^{51,70,77,93,94} under other lysis conditions, these discrepancies may reflect the extractability of the potentially redistributed receptors under certain fractionation conditions. Because different methods of cell breakage and assay have been used, this issue has not been resolved. It remains to be determined if the site of action of PK-C in activating the NADPH-oxidase is direct phosphorylation of the plasma membrane-bound NADPH-oxidase or modification of a cytosolic regulatory component which could then act on the oxidase.

Regardless of the transport of PK-C in intact cells, which appears to require membrane binding in physiologic stimulation,⁹⁵ *in vitro* manipulation, based on these binding studies of phorbol ester, has reconstituted a functional activation of the NADPH-oxidase. Previous studies showed that the membrane-bound NADPH-oxidase may be activated in a broken cell system by the addition of AA to a cytosolic factor.⁹⁶⁻⁹⁸ Those studies were extended by characterizing the pathway of oxidase activation through the PK-C system. PMA alone was unable to stimulate the oxidase in disrupted neutrophils when tested at concentrations corresponding to ~50-fold the K_d for the phorbol ester receptor; however, under specific conditions appropriate for PK-C activation, namely inclusion of PMA, PS, ATP, and a cytosolic factor, the NADPH-oxidase was activated.⁹¹

In these PK-C reconstitution studies, separation of the cytosol fraction and a light membrane fraction, the site of NADPH-oxidase in prefractionated PMA-stimulated neu-

trophils,³³ enabled definition of the basic requirements of the reconstituted oxidase system. Each component of a reconstituted PK-C system was required for NADPH-oxidase expression. The dependence of oxidase activation on particular phospholipids paralleled that for PK-C studies in other tissues.⁹² The Ca^{2+} dependence of the reaction could only be demonstrated with the addition of EGTA. Residual Ca^{2+} in the cytoplasm and leached membrane Ca^{2+} was presumably sufficient for reconstitution of activity. The dose-response curve for PMA revealed a half-maximally effective dose of 1.1 nmol/L, which was consistent with the high affinity of PMA for PK-C⁹⁹ and the measured amount of PK-C in the cytosol preparations. When purified PK-C obtained from mouse brain and its co-factors were substituted for cytosol, the NADPH-oxidase was similarly activated. Quantitatively, the levels of PK-C in the cytosol corresponded with the added purified enzyme.

As noted, activation of NADPH-oxidase has been achieved in broken cell preparations by adding AA,⁹⁶⁻⁹⁸ oleic acid,¹⁰⁰ or sodium dodecyl sulfate (SDS)¹⁰¹ to the membrane-bound oxidase. In these studies, an additional soluble factor was required, but it does not appear to be PK-C, as originally suggested. AA, oleate, or other oxygenated fatty acid derivatives activate PK-C directly,¹⁰²⁻¹⁰⁴ and AA inhibits specific [³H]PDBu binding to the cytosolic neutrophil phorbol ester receptor, with a K_i of 38 μ mol/L and an ID_{50} of 190 μ mol/L (P.M. Blumberg and A.I. Tauber, unpublished observations), values similar to those obtained using partially purified PK-C from mouse brain.¹⁰⁵ The mechanism for NADPH-oxidase activation by arachidonate in the *in vitro* setting does not correlate with these observations, however, and suggests that either another metabolic pathway is used or stimulation occurs by a nonenzymatic process. In both AA and SDS NADPH-oxidase broken-cell-stimulated systems, the PK-C inhibitor isoquinolinesulfonamide had no significant effect, supporting pathways of AA and SDS oxidase activation independent from that of PMA.¹⁰⁶

The initial characterization of the cytosolic factor(s) for arachidonate stimulation of the oxidase has shown that although they are Mg^{2+} -dependent, phospholipid, ATP, and GTP are not involved.^{106,107} Curnutte and colleagues showed that their putative soluble factor had no PK-C activity, and in a patient missing this factor, the level of neutrophil PK-C was normal.¹⁰⁸ As in their previous report,¹⁰⁹ the patient did not respond to PMA, suggesting that the cytosolic factor was either independent of PK-C or played its role distal to PK-C activation. It is of interest that the multifunctional kinase activator, calmodulin, has been implicated in oxidase activation by AA¹¹⁰ and other soluble agonists,¹¹¹⁻¹¹³ but only one preliminary report has used a calmodulin inhibitor in the broken cell AA-stimulated system and shown a possible linkage.¹¹⁴ The role of calmodulin in neutrophil activation, and in particular, its relation to PK-C, is as yet unclear. Finally, the correlation between intact cell activation with AA and that observed with broken cells is poor in that the former is Ca^{2+} dependent and releases prelabeled [³H]AA whereas the latter system is Ca^{2+} independent and does not depend on a putative PLA₂ function for oxidase expression.⁸⁸

The data suggest distinct mechanisms by which the NADPH-oxidase is stimulated in intact cells and membrane fractions by AA.

Although the precise stimulatory pathway or the physico-chemical correlates of AA or SDS activation have not been discerned, the evidence is compelling that AA and SDS activate the NADPH-oxidase by a PK-C-independent mechanism. Because the PK-C-stimulated oxidase is only 15% as active as that obtained with AA or SDS activation (which are comparable),¹⁰⁶ it may represent either a less efficiently reconstituted system or an ancillary arm of AA or SDS activation. The mechanism of the PK-C-independent activation may suggest the properties required for oxidase expression. One model for oxidase expression is based on the movement and juxtaposition of oxidase components for electron transfer from NADPH to oxygen. Badwey and colleagues, in studies with intact cells, first suggested that AA may activate the respiratory burst by effecting membrane fluidity changes in the locale of the NADPH-oxidase,¹¹⁵ and Bromberg and Pick postulate that AA and SDS activate the oxidase by their detergent properties.¹⁰¹ The physiological correlation to the PMA/PK-C system is that phosphorylation of a component (or possibly an intermediary mediator) affects the stereo-relationship of oxidase components, resulting in effective juxtaposition for electron transfer.

PUTATIVE PK-C PHOSPHORYLATED PRODUCTS

The roles of neutrophil protein phosphorylation have been examined with regard to the activation of the respiratory burst, since increased kinase or phosphatase activity with concomitant phosphorylation or dephosphorylation of target proteins represents an important regulatory mechanism in a variety of mammalian cells. Earlier work has identified human neutrophil protein kinases and phosphatases, but their functional roles have not been definitively determined.¹¹⁶ This issue may now be reexamined with our new understanding of PK-C and with the use of well-defined subcellular fractions.

Recognition of relevant phosphate acceptor proteins is one of the major problems for defining the effects of protein kinases in general. When tested in cell-free systems, PK-C shows broad substrate specificity and phosphorylates many endogenous unidentified proteins in most tissues. Apparently, protein kinases C and A often use the same phosphate acceptor proteins.^{117,118} For example, both enzymes phosphorylate, to various extents, seryl and threonyl residues in many membrane-bound proteins. A preliminary analysis suggests that the sites of phosphorylation overlap to some extent, but several seryl and threonyl residues are phosphorylated specifically by PK-C. The relative reaction velocities differ among the substrate proteins used, and the seryl and threonyl residues vary in amount and location. When H1 histone is used as a model substrate, the biochemical specificity is well illustrated: protein kinase A greatly favors a seryl residue (serine-38) located near the N-terminus, whereas protein kinase C rapidly phosphorylates both seryl and threonyl residues located near the C-terminus.^{119,120} Isolation of PK-C from bovine¹²¹ and rabbit¹²² neutrophils was

recently reported. Activity and biochemical characterization appear to correspond closely to PK-C preparations from other tissues.¹⁶

The role of PK-C-mediated protein phosphorylation in the activation of NADPH-oxidase has been studied by several groups using different neutrophil sources and techniques for demonstrating phosphorylated products. Not surprisingly, the reported findings show discrepancies and inconsistencies, making a coherent statement as to the identification of PK-C dependent phosphorylations impossible. The two most important lines of evidence that the identification of such products is crucial come from (a) the previously mentioned studies indicating that a functional oxidase arises from the reconstituted PK-C system, and (b) the recent demonstrations that both autosomal recessive¹²³ and X-linked¹²⁴ CGD patients lack phosphorylation of proteins of 44 to 48 kd. Although X-linked CGD has been associated with a defective or absent low potential b cytochrome,^{10,125} the molecular basis of autosomal CGD has not previously been established. The discrepancy that Segal does not find a phosphorylation defect in his X-linked CGD patients, but finds such a defect only in autosomal cases,¹²³ remains to be resolved. Heterogeneity of CGD defects is well known^{3,33} and the recent cloning by Orkin and co-workers of a missing protein found in some X-linked CGD cases that has no homology with known cytochromes has even raised uncertainty as to the significance of the b cytochrome as a molecular marker of the X-linked disease.¹²⁶ The tantalizing reports of Segal¹²³ and Babior¹²⁴ need to be pursued, with identification, isolation, and characterization of the putative crucial phosphorylated proteins in normal cells.

Previously, a major difficulty appears to have arisen from the use of insufficiently defined systems for analysis.^{124,127} Because the NADPH-oxidase has been localized to the plasma membrane, it seems appropriate to study the phosphorylated products at this site, or better, in a partially purified oxidase preparation. Recently, Rossi and colleagues examined such a preparation from pig neutrophils; the NADPH-oxidase was solubilized and filtered over Ultragel. Rossi found several phosphorylated products, but a 31.5-kd protein was prominently phosphorylated by an apparent PK-C-mediated mechanism,¹²⁸ and he suggested that this product was a component of the previously characterized oxidase.¹²⁹ Sha'afi's group also identified a 31-kd phosphorylated product in PMA-stimulated rabbit neutrophil plasma membranes, yielding a small, but consistent image. This protein was the only one identified as exclusively found in plasma membranes but was not further characterized.¹³⁰ Another protein of 50 kd appears to be a major PK-C substrate; while it correlates closely with degranulation, its relation to the activation cascade has not been further elucidated.^{130,131} An early study of a reconstituted PK-C system incubated with disrupted neutrophils identified major phosphorylated membrane-bound products of 38 and 34 kd, but the relationship of these products to oxidase activation was not investigated.⁵⁰ Babior found a 22-kd protein exclusively in the particulate fraction of disrupted human neutrophils prestimulated with PMA.¹³² Romeo's group, using

enucleated, granule-free cytoplasm ghosts (cytosol-free) found six PK-C-mediated phosphorylated products, but did not attempt to relate these targeted proteins to oxidase function.¹³³ One of these products is a 47-kd protein. An earlier Romeo study of human neutrophils could not distinguish PK-C specifically labeled proteins primarily because of the undifferentiated system of analysis used.¹³⁴ Okamura and colleagues attempted to relate this 46-kd species (probably two proteins with distinct pIs of 7.4 and 6.4) to NADPH-oxidase activation.¹³⁵ This study, using guinea pig neutrophils, found the 46-kd proteins in a membrane precipitate, but again their relation to oxidase activation was not established. We find the claim interesting only in light of the corresponding size of the missing proteins reported in the CGD studies of Segal and Babior.^{123,124}

Other activators of PK-C have also been used to examine phosphorylation products of the human neutrophil. Using preloaded cells stimulated with OAG, and two-dimensional gel electrophoresis, Fujita and co-workers observed four prominent images, but did not attempt to link any of these to oxidase activation.¹³⁹ Berkow and Kraft recently compared PMA- and Bryostatin-stimulated neutrophils and found unique products of 93, 88, 66 and 45 kd in a one-dimensional system⁴⁷; Bryostatin induced phosphorylation of an additional protein of 70 kd. The similar pattern supports the same mechanism of cell activation by these stimuli.

These previous studies alerted workers to the problems of (a) using a prelabeled intact cell system that cannot distin-

guish those products crucial to oxidase expression from other stimulated activities; (b) the high number of competing, non-PK-C kinase activities, which are not controlled in intact cell systems; (c) the complexity of phosphorylation patterns if purified membranes from the same animal or human source are not used; and (d) the necessity of relating phosphorylated products directly to oxidase expression. New approaches to defining the mechanism of PMA/PK-C-mediated activation of NADPH-oxidase must use a minimal system to reduce the number of phosphorylated products for study. Purified PK-C incubated with purified membranes containing the oxidase offers the opportunity to examine this process with minimal competing reactions and the fewest selected phosphorylation targets. Corresponding kinetics, inhibitor studies and PK-C specificity should identify those products(s) crucial for NADPH-oxidase activation by the PK-C system, which can then be confirmed with studies of CGD cells and corresponding studies with intact cells. Once identified, characterized, and isolated, the NADPH-oxidase activation system may be dissected and then reconstituted. Although this is an ambitious undertaking, the reward for tracing this receptor-ligand-coupled reaction is understanding of a major mechanism of signal transduction.

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Diversity of the Effect of Recombinant Tumor Necrosis Factors α and β on Human Myelogenous Leukemia Cell Lines

By Miloslav Beran, Borje S. Andersson, Peter Kelleher, Kathleen Whalen, Kenneth McCredie, and Jordan Gutterman

In vitro characteristics of response to recombinant tumor necrosis factors α (rTNF- α) and β (rTNF- β) were studied in six human myelogenous leukemia cell lines. Heterogeneity of the response to rTNF was observed, and one line (K562) was resistant. No enhancement of cell growth was noted in any cell line. The dose-response curves for rTNF- α were characteristically sigmoid, the maximum inhibitory effect occurring between 25 and 200 ng/mL. Nonresponsiveness within this range indicated resistance that could not be overcome, even with very high doses of rTNF- α . A similar response of sensitive and resistant lines occurred after exposure to rTNF- β . The clonogenic cells were more sensitive than the overall population to the action of

rTNF- α , and prolonged exposure was necessary for manifestation of the effect. Concomitant exposure to recombinant interferon- α (rIFN- α) increased the response of two cell lines to rTNF- α , but no clear synergistic action could be demonstrated. The addition of rIFN- γ was without effect. Variations in the rTNF- α -induced proliferative response could not be explained by differences in the number of binding sites per cell or their affinity for rTNF- α . That the clonogenic cells showed a higher sensitivity than the whole population might indicate a preferential effect on more primitive, actively proliferating cells with high growth potential.

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TUMOR NECROSIS FACTOR (TNF)^{1,2} and lymphotoxin³⁻⁵ were originally described as products of stimulated monocytes-macrophages and lymphocytes, respectively. These lymphokines are characterized in vitro by their lytic or antiproliferative effect on certain transformed cell lines.⁶⁻¹² In vivo they induce hemorrhagic necrosis of both autologous and heterotransplanted tumors.^{1,8,13,14} In numerous studies, there have been reports on variations of the in vitro activity of TNF in different animal and human cell lines,¹⁻¹⁵ but no myelogenous leukemia lines have been studied so far. Little is known about the modes of action of TNF.^{2,15,16} Only recently, because of the availability of pure molecules,¹⁷⁻²⁰ can the mechanism of action be properly studied. It was recently reported that recombinant TNF (rTNF) does inhibit growth of certain tumor cell lines while being indifferent or stimulatory to others,¹² and a similar observation was made with purified lymphotoxin.⁷

The activity of these lymphokines in normal and malignant human hematopoiesis is largely unknown at present. TNF is produced by both normal and malignant monocytes-macrophages²⁰⁻²² and natural killer (NK) cells.²³ Thus, its action on human leukemic cells is of particular interest since we have observed inhibition of leukemic clonogenic growth by human macrophages²⁴ and NK cells.²⁵ Myelogenous leukemia is a heterogeneous disease, both morphologically and cytogenetically.²⁶ We were interested in whether this heterogeneity is reflected in the in vitro response to TNFs. Of particular interest to us was the role of TNF receptors and the relation of the TNF response to the phenotypic properties of the cell lines. We therefore investigated the following aspects of cell-TNF interactions: (a) the dose response of leukemic clonogenic cells to rTNF- α ^{12,20} and lymphotoxin or TNF- β (rTNF- β)^{12,19}; (b) the action of rTNF- α on clonogenic cells as compared with the whole leukemic cell population; and (c) the receptor density and binding affinity to cellular receptors of radiolabeled rTNF- α .

Interferons have had antiproliferative activity in some leukemic cell lines studied (M. Beran, unpublished observations), and synergism between interferons and TNFs has been reported in some cell systems.^{7,9,10} Therefore, we also investigated a possible enhancement of the TNF response in TNF-sensitive and TNF-resistant leukemic cell lines by rIFN- α and IFN- γ . Because clonogenic cells are responsible

for the continuous growth of a tumor, we primarily chose to study the effect of rTNFs on these cells rather than the whole population of tumor cells.

MATERIALS AND METHODS

Lymphokines. Recombinant technology-produced interferon- γ (rIFN- γ , specific activity, 4×10^7 U/mg), rTNF- α (specific activity, 3.7×10^7 U/mg), and lymphotoxin or rTNF- β (specific activity, 2.5×10^7 U/mg) were all gifts from Genentech (San Francisco). Recombinant rIFN- α , a gift from Hoffman LaRoche (Nutley, NJ) had a specific activity of 2.0×10^8 U/mg. Stock preparations were stored at 4°C in sterile vials. Dilutions were made immediately prior to use in sterile phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum (FCS). The amount of rTNF- α and rTNF- β is given in nanograms rather than in units to avoid controversy related to differences in the sensitivity of various assay TNF systems.

Leukemic cell lines. Six different cell lines²⁷⁻³⁰ derived from patients with acute myelogenous leukemia (AML) or blastic crisis of chronic myelogenous leukemia (CML-BC) were used in the present study (Table 1). One additional subline of KBM-3, rendered resistant to Adriamycin (KBM-3/Adria), was also used.

All leukemic cells were maintained as suspension cultures in Iscove's modification of Dulbecco's medium (IMDM) (Irvine Scientific, Santa Ana, CA) supplemented with 10% to 15% FCS. They were used for experiments when in exponential growth phase.

Clonogenic assay. All leukemic cell lines that were used produced clones in semisolid media, without an external source of specific growth factors. The cloning system used was a standard, single-layer agar media culture system. Cells were suspended in IMDM supplemented with 15% FCS, L-glutamine, penicillin, streptomycin, and 0.3% agar (Bacto-Agar, GIBCO, Grand Island, NY). One milliliter of media, containing 5 to 10×10^3 cells, was added to

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Table 1. Myelogenous Leukemia Cell Lines

Cell Line	Origin	Morphological Phenotype	Passage	Reference
K-562	CML-BC	Myeloid, poorly differentiated	Unknown	27
HL-60	AML-M3	Promyelocytic	Unknown	28
EM-2	CML-BC	Myeloid	Unknown	29
KBM-3	AML-M4	Monocytic	20-150	Andersson and Beran (submitted for publication)
KBM-5	CML-BC	Myelomonocytic	50-100	Andersson and Beran (submitted for publication)
KBM-7	CML-BC	Myeloid	20-40	32

35-mm tissue culture Petri dishes (Nunc, Denmark) and allowed to solidify. Cultures were then incubated at 37 °C in a fully humidified atmosphere of 5% CO₂ in air. After 7 days, clones containing more than eight cells were scored. All cultures were made in triplicate for each experiment.

Normal human bone marrow granulocyte-macrophage progenitors (GM-CFCs). Bone marrow was obtained from six normal volunteers and light-density fraction, devoid of erythrocytes and granulocytes obtained using one-step Hypaque-Ficoll (density 1.080 g/mL). In accordance with institutional guidelines, all patients and volunteers were advised of bone marrow aspiration procedures and attendant risks and gave informed consent. After removal of adherent cells by incubation in plastic bottles for 2 hours with 20% FCS, the nonadherent cells were used for comparative study on the effect of rTNF- α . Cells (1×10^5 mL) were suspended in IMDM supplemented with 20% FCS, 0.3% agar, and increasing concentrations of rTNF- α (1×10^{-2} to 1×10^3 ng/mL). One milliliter was plated in 35-mm Petri dish on the top of 1 mL of media supplemented with 20% FCS, 0.5% agar, and 10% (vol/vol) of human placenta-conditioned medium (HPCM) as the source of colony-stimulating factor.^{31,32} Triplicate cultures were set up for each experimental point and incubated at 37 °C, 5% CO₂ in air for 6 days. Thereafter, clones containing ≥ 50 cells were scored, surviving fractions were calculated, and data were plotted as dose-response curves. The maximal achievable inhibition of clonal growth and the lowest dose of rTNF- α producing maximal inhibition was determined.

Assay on the effect of lymphokines in agar-media cultures. Recombinant IFN- α or IFN- γ , rTNF- α , and rTNF- β or their combinations were added in increasing concentrations to cells in semisolid cultures. In this system, the cells were continuously exposed at relatively low density to particular lymphokines. After the clones produced by surviving clonogenic cells were scored, survival curves were constructed by comparing the numbers of clones in experimental cultures with those from control plates. Surviving fractions were calculated. The results, expressed as means $\pm$ SD of triplicate cultures, were plotted on a log/log plot as dose-response curves.

Effect of rTNF- α on clonogenic cells in suspension cultures of leukemic cells. Short-term cultures were carried out in IMDM supplemented with 15% FCS. Cells (5×10^4 to 3×10^5 /mL) were added along with rTNF- α and incubated at 37 °C, 5% CO₂ in air. After 1 hour to 4 days, the cells were pelleted at 150 g for 10 minutes, the supernatant was discharged, and the cells were washed twice in warm PBS to remove unbound lymphokines. Cells were counted in a counting chamber, and viability was determined by trypan blue exclusion test. A fraction of cells was plated for determination of the surviving clonogenic cells. All suspension cultures were done in duplicate. From each culture, clonogenic assays were performed in triplicate as described above.

Radioiodination of rTNF- α . Radioiodination of rTNF- α was performed by the iodogen method³³ as described by Aggarwal and colleagues.³⁴ In brief, 10 μ g of rTNF- α in 0.1 mol/L of phosphate buffer (pH 7.4) was added to 0.5 mCi Na¹²⁵I in a tube coated with 5 μ g iodogen for 10 minutes at 4 °C. The reaction was stopped by

removing soluble material from the tube, and labeled rTNF- α was separated from free iodine by filtration on Sephadex G-10 equilibrated with 0.1 mol/L of phosphate buffer containing 0.1% gelatin and 0.05% Tween 20. ¹²⁵I-labeled TNF had a specific activity of ~ 630 Ci/mmol and gave a single band of 17,000 daltons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). ¹²⁵I-labeled TNF retained 80% to 90% of its biological activity as assayed by the lysis of actinomycin D-treated mouse L929 cells.³⁵

TNF binding assays. Binding of ¹²⁵I-labeled TNF was performed as described for ¹²⁵I-labeled rIFN- α .³⁶ Cells were harvested by centrifugation at $1 \times 10^3 \times g$ for 10 minutes, resuspended in medium, and again pelleted. The cell pellet was resuspended in RPMI 1640, containing 0.1% bovine serum albumin (BSA), to a concentration of 1 to 2×10^7 cells/mL. Cell suspensions were incubated with either ¹²⁵I-labeled rTNF- α only for total binding or ¹²⁵I-labeled rTNF- α plus excess unlabeled ($\times 100$) rTNF- α to assay for nonspecific binding. After incubation for 2 hours at 24 °C 100- μ L aliquots were removed in triplicate and layered on cold (4 °C) sucrose double gradients (0.15 mol/L/0.30 mol/L). The tubes were centrifuged for 15 seconds at $13 \times 10^3 g$ in a Beckman tabletop microfuge (model no. 152) and immediately frozen in an alcohol-dry ice slurry. Bound (pelleted) and free (supernatant) ¹²⁵I-labeled rTNF- α were separated by cutting off the bottom part of the microtube. Specific binding was determined by calculating the difference between the total and the nonspecific binding. Calculated values of the number of binding sites per cell were obtained by Scatchard analysis of equilibrium binding data.³⁷ Student's *t* test was used to evaluate the significance of the results.

RESULTS

Effect of rTNF- α on clonogenic cells. An appreciable heterogeneity in the cloning ability of six different human myelogenous leukemia cell lines was observed in the continuous presence of rTNF- α in the culture medium (Fig 1, Table 2). The sensitivity range of GM-CFCs is presented for comparison (Table 2). The clonogenic cells of K562 line proved resistant to rTNF- α concentrations up to 5×10^3 ng/mL. In responsive cell lines, the dose-response curves on a log/log plot were sigmoid.

In all cell lines tested, a maximal inhibition of clonogenic growth was observed after the addition of ≤ 100 ng/mL rTNF- α . Further increase of the TNF concentration resulted in either a plateau or a decrease in the degree of inhibition. In sensitive cell lines, the major differences were observed after doses < 25 ng/mL (Fig 1). The KBM-3 cells proved exquisitely sensitive, significant inhibition being noted as reproducible at doses of 1×10^{-3} ng/mL; 10 ng/mL inhibited 99.8% of clonogenic cells. Stimulation of colony growth was not observed with any cell line in the dose range between 1×10^{-4} and 1×10^4 ng/mL.

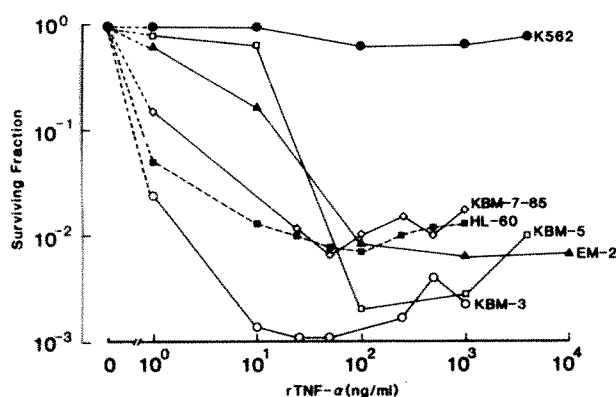


Fig 1. Sensitivity of clonogenic cells from myelogenous leukemia cell lines to increasing doses of recombinant tumor necrosis factor- α (rTNF- α). Each point represents the mean of three experiments. (SD signs are omitted for simplicity.)

Comparison of the activity of rTNF- α and rTNF- β in leukemic clonogenic assay. In two cell lines selected for differences in their response to rTNF- α , the effect of rTNF- β was studied in the clonogenic assay. (Fig 2). Similar results were obtained with both lymphokines. In the sensitive (KBM-5) cell line, rTNF- β (lymphotoxin) proved less active on a wt/vol basis. In K-562 cells, the same resistance was shown to rTNF- α and rTNF- β .

Effect of rTNF- α on the growth of myelogenous leukemia cells in suspension cultures. In two rTNF- α -sensitive cell lines, we studied the effect of cell density on the inhibitory effect of rTNF- α . In HL-60 cells, the growth inhibition was more pronounced in cultures initiated at lower initial cell concentrations (Table 3). Evaluated by total cell numbers as endpoint, the phenomenon was found only in moderately sensitive HL-60 cells. In highly sensitive KBM-3 cells between 73% and 77% inhibition was found after 3 days of growth and the degree of growth inhibition was independent of the initial cell density. The cell viability assessed by trypan blue exclusion test was slightly but significantly ($P < .05$) lower in the presence of rTNF- α .

Effect of rTNF- α on clonogenic cells compared with the whole cell population in suspension cultures. HL-60 cells were grown in suspension culture at two different seeding cell densities, in the presence or absence of 500 ng/mL of rTNF- α . The results summarized in Table 4 demonstrate

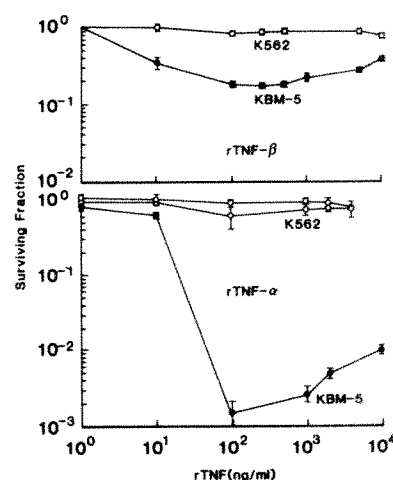


Fig 2. Comparison of the effect of recombinant tumor necrosis factor α (rTNF- α) and lymphotoxin (rTNF- β) on clonogenic cells from two myelogenous leukemia cell lines. Each point represents the mean $\pm$ 1 SD of quadruplicate cultures.

that rTNF- α preferentially inhibits in vitro growth of clonogenic cells as compared with the whole cell population. Similar results were obtained with the highly sensitive KBM-3 cell line (data not shown).

Short-term effect of r-TNF- α on clonogenic cells. We further investigated whether clonogenic cells can be affected by short-term exposure (2 hours) to rTNF- α in doses approximately five to ten times higher than doses causing maximum inhibition of clonal growth in semisolid media. The results observed in two sensitive cell lines are summarized in Table 5. Two-hour exposure inactivated a significant number of clonogenic cells in both cell lines. Viability of cells of both lines, assessed by trypan blue exclusion test, was unaffected by the 2-hour exposure (96% to 98%).

Effect of combination of rTNF- α with rIFN- α or rIFN- γ in clonogenic growth. The addition of increasing doses of rIFN- α or rIFN- γ to a standard dose of rTNF- α affected the rTNF- α -related inhibition only to a limited extent. The results shown in Table 6 show that rIFN- α , but not rIFN- γ , at very high doses (5,000 U/mL) slightly ($P < .05$) potentiated the inhibitory action of TNF in KBM-5 clonogenic cells. At this particular dose level, rIFN- α was not inhibitory itself (data not shown). rIFN- γ appears to decrease the

Table 2. Analysis of rTNF- α Binding to Myelogenous Leukemia Cells and Comparison to Their Sensitivity to rTNF- α in Clonogenic Assay

Cell Line	rTNF- α Sensitivity*	Lowest rTNF- α Dose Producing Maximal Inhibition (ng/mL)	K_d (nmol/L) ($\times 10^{-10}$)	Average No. of Binding Sites per Cell (r)
K-562	6.5×10^{-1}	100	4.654	1,388
KBM-3	1.0×10^{-3}	25	2.836	7,086
KBM-5	2.0×10^{-3}	100	4.907	6,983
KBM-3/Adr†	1.2×10^{-2}	100	2.408	3,030
Normal				
GM-CFC‡	$6.5 \times 10^{-1} - 1.9 \times 10^{-1}$	100-500	—	—

rTNF- α , recombinant tumor necrosis factor- α .

*Values represent the surviving fraction at the maximal achievable inhibition.

†The KBM-3 cell line was rendered resistant by intermittent in vitro exposure to adriamycin (Adr) over a 1-year period.

‡Range of six individual marrows.

Table 3. Growth of Myelogenous Leukemic Cell Lines in Suspension Cultures in the Presence of rTNF- α : Effect of Initial Cell Density on the Inhibition of Growth

Cell Line	Seeding Density ($\times 10^4$ /mL)	TNF (ng/mL)	Viability (%)	Viable Cells* ($\times 10^4$ /mL)	Inhibition* (%)
HL-60	5	0	99 $\pm$ 0	42 $\pm$ 7	66 $\pm$ 10
	5	500	99 $\pm$ 0	14 $\pm$ 4†	
	15	0	99 $\pm$ 0	123 $\pm$ 11	56 $\pm$ 10
	15	500	98 $\pm$ 1	54 $\pm$ 12†	
	30	0	99 $\pm$ 0	256 $\pm$ 26	46 $\pm$ 7‡
	30	500	99 $\pm$ 0	138 $\pm$ 18†	
KBM-3	5	0	96 $\pm$ 2	40 $\pm$ 12.0	
	5	500	84 $\pm$ 4	9.5 $\pm$ 2.7†	76 $\pm$ 6
	15	0	98 $\pm$ 1	136.0 $\pm$ 26.0	
	15	500	85 $\pm$ 5	30.0 $\pm$ 5.4†	79 $\pm$ 5
	30	0	97 $\pm$ 2	253.8 $\pm$ 18.7	
	30	500	83 $\pm$ 5	69.5 $\pm$ 10.1†	73 $\pm$ 4

rTNF- α , recombinant tumor necrosis factor- α .

Cells were grown in Iscove's modified Dulbecco's medium supplemented with 15% fetal calf serum and harvested after 3 days in culture.

*Values represent the means $\pm$ SD of six separate experiments.

†Difference between control and TNF containing cultures significant at $P = < .005$.

‡Significantly ($P < .005$) different from inhibition at density of 5×10^4 but not significantly different from density of 15×10^4 .

inhibitory effect of rTNF- α , which is in accord with its growth stimulatory action in the particular passages of these cell lines (data not shown). Similar results were obtained with the EM-2 cell line (Table 6), whereas no enhancement of TNF action by either interferon was found in the TNF-nonresponsive K562 cell line (data not shown).

Receptors for TNF in leukemic cell lines. To investigate whether the heterogeneity in the cellular response to rTNF- α could be due to the heterogeneity in rTNF- α binding sites, we examined the binding of radiolabeled rTNF- α to several cell lines that differed in their sensitivity to rTNF- α in the clonogenic assay (Table 2). The results indicate heterogeneity, both in binding sites per cell and in the affinity of binding sites for the ligand (Table 2). Among all cell lines tested, the correlation to the inhibitory action of TNF was difficult to establish. This indicates that the sensitivity of cells is not simply reflected in the number of binding sites or in ligand affinity, although there was a tendency toward a slightly higher number of binding sites in sensitive cells.

DISCUSSION

The present study revealed a considerable heterogeneity in the sensitivity to rTNF- α of clonogenic cells of six myelogenous leukemia cell lines. No enhancement of clono-

genic cell growth was noted, and one cell line (K562) proved resistant to TNF on continuous exposure of up to 5×10^3 ng/mL. The TNF-sensitive cells responded to increased TNF doses in a characteristic fashion, with a sigmoid dose-response curve on a log/log plot, irrespective of the degree of sensitivity. An important finding was that maximal inhibition was reached at a relatively low concentration of TNF. This may indicate a mode of action compatible with a saturable mechanism.

Analysis of rTNF- α binding properties failed to reveal a consistent pattern to which the sensitivity of cells could be related. This is in agreement with the earlier study of Umeda and co-workers,¹⁶ in which absorption techniques were used, and also with recently reported data on nonhematopoietic cell lines.¹² It suggests that although receptors may be necessary for mediation of TNF action, the magnitude of TNF action is determined by additional as yet unknown cellular mechanisms. The observation that IFN- γ failed to influence the TNF response in either TNF-sensitive or TNF-resistant cells is incongruous with recently published data.¹² In a very few cell systems, however, a synergism, mainly between lymphotoxin (rTNF- β) and IFN- α or IFN- γ , was observed.^{7,9-12} Thus, there may be tissue and cell lineage specificity. Some data indicate that IFN- γ increases the synthesis of TNF

Table 4. rTNF- α Inhibition of Myelogenous Leukemia Clonogenic Cells of HL-60 Cell Line

rTNF- α (ng/mL)	Cells Seeded		Cells Recovered*			
	Total Cells	L-CFC	Total Cells†		L-CFC	
	($\times 10^4$ /mL)	($\times 10^4$ /mL)	($\times 10^4$ /mL)	Inhibition (%)	($\times 10^4$ /mL)	Inhibition (%)
0	5	1.49	72 $\pm$ 0.9	—	21.3 $\pm$ 0.9	—
500	5	1.49	28 $\pm$ 0.7	61.1 $\pm$ 2.1	3.4 $\pm$ 0.3	84.0 $\pm$ 3.7‡
0	30	8.90	357 $\pm$ 21	—	62.5 $\pm$ 3.0	—
500	30	8.90	240 $\pm$ 30	32.8 $\pm$ 0.9	11.7 $\pm$ 7.0	81.3 $\pm$ 3.4‡

rTNF- α , recombinant tumor necrosis factor- α ; L-CFC, leukemic clonogenic cells.

*Cells were recovered after 3 days in suspension cultures.

†Values represent mean $\pm$ SD of triplicate cultures.

‡Significantly ($P < .01$) higher than inhibition of total cells.

Table 5. Mechanism of Action of rTNF- α on Myelogenous Leukemia Cell Lines: Acute Effect on Clonogenic Leukemic Cells

Cell Line	No. of Experiments	No. of Leukemic Clonogenic Cells*		
		Pretreatment	Posttreatment	Inhibition (%)
HL-60	6	6,631 $\pm$ 347	1,520 $\pm$ 144†	77 $\pm$ 2
KBM-3	6	1,998 $\pm$ 90	403 $\pm$ 102†	69 $\pm$ 3

rTNF- α , tumor necrosis factor α .

*Leukemic cells (5×10^4 /mL) were incubated in 1 mL of Iscove's medium, supplemented with 15% fetal calf serum (FCS) and 500 ng/mL of recombinant TNF at 37 °C. After 2 hours, the cells were washed twice in 10 mL of warm (39 °C) phosphate-buffered saline with 2% FCS to remove TNF. A portion of cells (10^4 /dish) was plated in agar-medium, and colonies of eight cells or more were scored 7 days later. Mean $\pm$ SD of six experiments.

†Significantly ($P < .01$) lower than pretreatment values.

receptors.³⁴ Because of the lack of correlation between TNF receptor numbers, binding affinity, and cell sensitivity, the role of such a mechanism in IFN-TNF cell interactions remains unclear.

Short-term (2 hours) exposure of sensitive leukemic cell lines to moderately high (up to 5×10^3 ng/mL) doses of rTNF- α had no effect on viability of leukemic cells as evaluated by dose-exclusion test. Such short exposure did, however, inhibit subsequent colony formation by clonogenic cells. Although these findings do not support a notion of a direct cytolytic action of TNF in the leukemic cell lines studied, inhibition of cell proliferation as the mechanism underlying its inhibitory action is suggested. This is further supported by the absence of decreased cell viability with increased inhibition of colony formation.

The finding that cell line HL-60 produces TNF when appropriately stimulated²⁰ is suggestive of the role of TNF in a self-regulatory mechanism. In our studies, we showed that the parental, undifferentiated cell line is a very sensitive target for rTNF- α . Whether other AML leukemic cell lines studied here produce TNF is not known and is currently being investigated, as is the relation of sensitivity to the degree of maturation. Production by tumor cells when induced to differentiate of a factor (TNF) that is highly inhibitory to proliferation of clonogenic tumor cells is an

intriguing phenomenon and, if verified in vivo, could have potential therapeutic implications.

Examination of a large number of both mouse and human¹⁻¹⁵ tumor cell lines has revealed considerable heterogeneity in their response to TNF. Because the initial studies used preparations of varying purities derived from various species, it was difficult to compare the relative sensitivities of cell lines used in the different experiments. Furthermore, the sensitivity could vary within each cell line depending on the number of passages and the laboratory in which it was maintained. It was difficult, therefore, to conclude whether a certain response to TNF is related to a particular malignant cell type. In a recent report, however, a heterogeneous response of both human and mouse tumor cell lines to pure rTNF- α was observed.¹²

The purpose of our studies was to examine rTNF- α and IFN- β response in several cell lines derived from one type of hematologic malignancy that had phenotypic features of myelogenous leukemia. We were particularly interested in this tumor type because a TNF message was derived from one AML cell line that was induced to differentiate along the monocytic pathway.²⁰ Thus, the possible antitumor effect could not only be compared with the degree of differentiation of a particular cell line but also with the response of normal hematopoietic progenitor cells. The heterogeneity in the observed response was considerable even within the myelogenous, leukemia-derived cell lines. One line, K562, was resistant to both rTNF- β and rTNF- α , whereas the other lines showed various degrees of sensitivity with no apparent correlation to morphological type. Interestingly, HL-60 was highly sensitive to the antiproliferative effect of rTNF- α , and it was also shown that clonogenic cells—presumably a more primitive category of cells—were more sensitive than the overall population to TNF's inhibitory action. The rTNF- α and rTNF- β resistance of K562 is of particular interest because this cell line is extremely sensitive to natural killer (NK) cell-mediated killing.

Our finding is incongruous with results of a recent report suggesting that rTNF- α may be a major mediator of NK cell action.²⁴ The data show that most myelogenous leukemia cells are very sensitive to TNFs. With the exception of K-562, their sensitivity to rTNF is appreciably higher than that of GM-CFCs³⁸ and comparable to that of clonogenic cells derived from primary acute myelogenous leukemias.³⁸ The availability of cell lines with a defined degree of sensitivity is important for future studies of the mechanism of action and development of resistance to TNFs.

Table 6. Effect of rIFN- α and rIFN- γ on rTNF- α -Induced Inhibition of the Clonogenic Growth of Two Myelogenous Leukemia Cell Lines

Cell Line	rTNF- α (ng/mL)	rIFN- α (U/mL)	rIFN- γ (U/mL)	Survival* (%)
KBM-5	100	0	0	12 $\pm$ 1
	100	1,000	0	8 $\pm$ 4
	100	5,000	0	3 $\pm$ 1†
	100	0	0	9 $\pm$ 2
	100	0	1,000	12 $\pm$ 1
	100	0	5,000	16 $\pm$ 4†
EM-2	100	0	0	10 $\pm$ 1
	100	1,000	0	7 $\pm$ 2
	100	5,000	0	4 $\pm$ 1†
	100	0	0	3 $\pm$ 1
	100	0	1,000	6 $\pm$ 2†
	100	0	5,000	ND

rIFN- α , recombinant interferon- α ; rTNF- α , recombinant tumor necrosis factor- α ; rIFN- γ , recombinant interferon γ ; ND, not done.

*Values represent mean $\pm$ 1 SD of triplicate cultures.

†The overall response differs significantly ($P < .05$) from the response with rTNF- α only.

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Erythrocyte Deformation in Shear Flow: Influences of Internal Viscosity, Membrane Stiffness, and Hematocrit

By Kazunori Kon, Nobuji Maeda, and Takeshi Shiga

The effect of shear force (depending on shear rate and viscosity of extracellular medium) and hematocrit of RBC suspension on RBC deformation was studied quantitatively using a cone-plate rheoscope with various kinds of cells, ie, partially hemolyzed (PH) cells, density-fractionated intact cells, and diamide-treated cells. The deformation index (DI) of ellipsoidally deformed cells was shown to be a function of $\beta\dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^\alpha$, where $\dot{\gamma}\eta_{ex}$ is applied shear stress, η_{ex} and η_{in} are external and internal viscosities, respectively, and α and β are adjustable parameters related to the membrane viscoelastic properties. The increase of suspen-

sion viscosity at higher hematocrits (Hts) generally enhanced the ellipsoidal deformation of cells, in the same manner as increasing the suspending medium viscosity of a diluted cell suspension. The suppressing effect on cell deformation appeared above a certain Ht. When intact cells were mixed with glutaraldehyde-treated, hardened cells, the ellipsoidal deformation of intact cells was disturbed. The suppression of deformation probably occurred through disturbance of laminar flow-lines around intact cells.

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IT IS WELL KNOWN that blood viscosity, as well as the viscosity of red cell suspension decreases with an increase in a shear rate, because the deformation and orientation of cells reduces hydrodynamic resistance against shear flow.¹ At high shear stresses, RBCs stably orient and elongate, accompanying membrane tank-treading about the cell interior.² The cellular deformation in shear flow is frequently treated by the analogy with that of liquid droplet^{3,4} as described by Taylor⁵ and Cox,⁶ ie, the elongation of cells is enhanced by the increase in viscosity of suspending medium and/or in shear rate. Although the major determinants of the ability of deformation (deformability) of RBCs (ie, intracellular viscosity, membrane stiffness, and cell geometry) have been revealed,⁷⁻¹⁰ the quantitative contribution of these determinants to deformability is not yet clear. In the present study, we assessed quantitatively, over a wide range of shear stress, the influence of intracellular viscosity and membrane stiffness on cellular deformation.

In addition to single cell deformability, to study rheological phenomena of circulating blood, the role of hematocrit should be investigated as an influencing factor on cellular deformation. Rheoscopy¹¹ allows us to observe the cell deformation directly and to measure the degree of cell deformation in a diluted suspension. Direct observation of individual cells is difficult, however, in the suspension of physiological hematocrit (Ht) value because of reduction in light intensity for microscopic observation. Therefore, we prepared partially hemolyzed cells (PH cells) to study the influence of Ht on cellular deformation, ie, we replaced intracellular hemoglobin of intact cells with dextran to increase the transparency of cells and maintain intracellular viscosity.

This article describes: (a) the contribution of viscosity of suspending medium and shear rate on the degree of deformation of various kinds of cells, such as PH cells having different intracellular viscosity, density-fractionated intact cells, and diamide-treated intact cells; (b) the influence of Ht on the cellular deformation over a wide Ht range (from 4% to 55%); and (c) the behavior of intact cells in a mixed suspension of intact cells and glutaraldehyde-treated, hardened PH cells. In these studies, the cell deformation in shear flow is discussed on the basis of an empirical variable depending on the applied shear stress, the internal and external viscosities, and two parameters related to membrane stiffness.

MATERIALS AND METHODS

Preparation of PH Cells

Pink ghosts were prepared by hypotonic hemolysis in a preliminary experiment, but ellipsoidal deformation under uniform shear flow was disturbed. Therefore, we prepared PH cells containing an appropriate amount of dextran by the following procedures.

Freshly drawn heparinized blood from healthy donors was centrifuged to remove plasma and buffy coat. The packed cells were then washed three times with an isotonic HEPES buffer (115 mmol/L of NaCl, 50 mmol/L of HEPES, 6 mmol/L of glucose, 5 mmol/L of KCl, pH 7.4). The washed cells were suspended in the isotonic HEPES buffer containing 38 μ mol/L of L- α -lysophosphatidylcholine, palmitoyl (LPC, Sigma Chemical, St Louis) at a Ht of 45% (the RBCs changed from discocytes to spherocytocytes in the presence of LPC). After the suspension was allowed to stand for 5 minutes on ice, it was centrifuged to obtain packed LPC-treated cells. Two kinds of PH cells, having low internal viscosity (light PH cells) and high internal viscosity (heavy PH cells), were prepared by incubating LPC-treated cells in hypotonic dextran solutions as follows.

Light PH cells. In step 1, 1 mL of the LPC-treated packed cells was suspended in 9 mL of ice-cooled hypotonic HEPES buffer (10 mmol/L of HEPES, 1 mmol/L of KCl, 1 mmol/L of glucose, 2 mmol/L of EGTA, 5 mmol/L of MgSO₄, 0.8 mmol/L of ATP; pH 7.4, 30 mOsm/kg) containing 7% Dextran T-40 (Pharmacia Fine Chemicals, Uppsala, Sweden) and was incubated for 5 minutes on ice with gentle stirring (designated as step-1 suspension). In step 2, 9 mL of the same hypotonic 7% dextran solution was added to the step-1 solution, and the suspension was stirred gently for 5 minutes. In step 3, to return the isotonicity, 1.44 mL of a hypertonic HEPES buffer (1,150 mmol/L of NaCl, 500 mmol/L of HEPES, 60 mmol/L of glucose, 50 mmol/L of KCl; pH 7.4) was added to the

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step-2 suspension. In step 4, to remove LPC from cells, 18 mL of the isotonic HEPES buffer containing 1.5% bovine serum albumin (BSA, fraction-V, purchased from Armour Pharmaceutical, Kankakee, IL) was added to the step-3 suspension, followed by incubation of the suspension for 1 hour at 37 °C. During the incubation, resealing of cells was accomplished. In step 5, the resulting PH cells were washed once with an isotonic 7% dextran solution (100 mmol/L of NaCl, 50 mmol/L of HEPES, 6 mmol/L of glucose, 5 mmol/L of KCl; pH 7.4) to remove a minute amount of membrane fragment and washed twice with the isotonic HEPES buffer.

Heavy PH cells. The above procedures for the preparation of light PH cells were followed with a minor modification: In step 2, instead of the hypotonic 7% dextran solution, 9 mL of the hypotonic HEPES buffer containing 30% dextran was added to the step-1 suspension.

Preparation of Density-Fractionated Intact Cells

Intact cells were fractionated by density-gradient centrifugation.¹² An isotonic suspension of Percoll (Pharmacia) in the HEPES buffer was used: 75.6% Percoll to obtain low-density cells and 82.8% Percoll to obtain high-density cells. One milliliter of packed cells was poured on 13.5 mL of Percoll medium and then spun at 7,000 g for 20 minutes at 20 °C. The low-density cells (10% to 15% of all cells) gathered in the upper half of the tube (in 75.6% Percoll) while the high-density cells (30% of all cells) sedimented in the lower half of the tube (in 82.8% Percoll). The low-density cells and high-density cells can be assigned to young and aged cells, respectively.^{13,14} The fractionated cells were washed three times with the isotonic HEPES buffer to remove Percoll. The fractional percentage was calculated on the basis of hemoglobin concentration.

Preparation of Diamide-Treated Hardened Cells (Diamide-Treated Cells)

The fractionated low-density cells were suspended in the isotonic HEPES buffer and incubated with various concentrations of diamide (diazene dicarboxylic acid bis(*N,N*-dimethylamide, Sigma) for 30 minutes at 37 °C (Ht 10%).¹⁵ After incubation, cells were washed three times with the isotonic HEPES buffer.

Preparation of Glutaraldehyde-Treated, Hardened PH Cells (GA-Treated PH Cells)

Light PH cells were suspended in the isotonic HEPES buffer containing 0.1% glutaraldehyde at a Ht of 10% and incubated for 30 minutes at 37 °C with gentle stirring. To remove unreacted glutaraldehyde, cells were washed three times with the isotonic HEPES buffer.

Determination of Mean Corpuscular Volume (MCV)

MCV was determined from the number of cells measured by an automatic counter (Model CC-110, Toa Electric, Tokyo), and the packed cell volume was determined by the method of England and Down,¹⁶ as follows. Cells were washed with the isotonic HEPES buffer containing 1.5% BSA and suspended in the same solution. Ten microliters of ¹³¹I-labeled BSA solution (150 μ Ci/mL) were added to 5 mL of the cell suspension (Ht 50%). The radioactivities of supernatant and sedimented fraction were measured by an autowell γ counter (ARC-605, Aloka, Tokyo) after centrifugation for 10 minutes at 6,000 g with a microhematocrit tube using a microhematocrit centrifuge (Model KH-120, Kubota, Tokyo) and the packed cell volume was calculated after the volume of medium trapped among cells was corrected.

The Ht value of the cell suspension was determined after correcting the trapped medium volume. For the GA-treated PH cell

suspension, the Ht was calculated from the number of cells in the suspension and the MCV of original cell before GA treatment.

Electrophoretic Analysis of Membrane Proteins

The polyacrylamide gel electrophoresis (PAGE) in 1% sodium dodecyl sulfate (SDS) was performed on a slab gel.¹⁷ The densitometric scanning of Coomassie brilliant blue R-250-stained gel was performed by a densitometer (Dual-wavelength TLC Scanner, CS-900, Shimadzu, Kyoto, Japan). After diamide treatment, the degree of cross-linking of membrane proteins was estimated by the method of Maeda and colleagues.¹⁵

Determination of Amounts of Membrane Lipids

To estimate the loss of membrane lipids during the preparation of PH cells, the amounts of cholesterol and total phospholipids were determined by the method of Zlatkis and Zak¹⁸ and Bartlett,¹⁹ respectively.

Measurement of Cellular Deformation

Cellular deformation was observed at 25 °C with a cone-plate rheoscope¹¹ constructed in our laboratory^{15,20} combining an inverted microscope (Model IMT, Olympus Optics Co., Tokyo), a transparent 0.8° cone-plate viscometer (Model B, Tokyo Keiki) and a flashlight (Model MS-230, Sugawara, Tokyo). Two experimental protocols were used as follows. First, cells were suspended in isotonic dextran solutions to make a Ht of 0.4% to observe cell deformation in a diluted suspension. The shear rates were changed stepwise in the range of 75.2 to 752 s⁻¹. The dextran concentration was varied in the range of 7.5% (4.0 cP) to 20% (18.3 cP). Second, for observation of the cellular deformation at higher Hts, cells were suspended in either isotonic dextran solutions (6% or 10%) or plasma with varying Ht (4% to 55%), and a constant shear rate (752 s⁻¹) was applied.

The osmolality of dextran solutions was adjusted with NaCl by using a Halbmikro osmometer (Type M, Knauer, FRG) after dissolving an appropriate amount of dextran in a hypotonic HEPES buffer (50 mmol/L of HEPES, 5 mmol/L of KCl, 6 mmol/L of glucose). The degree of deformation of stably oriented and deformed cells was expressed by the DI, defined by $DI = (L - B)/(L + B)$, where L and B are the lengths of long and short axes of ellipsoidally deformed cells.³ For each sample, DIs of 60 to 80 cells were measured.

Measurement of Viscosity

To measure the viscosity of hemolysate and cell suspension, a cone-plate viscometer (Model E, Tokyo Keiki) was used at 25 °C. For measurement of intracellular viscosity, the hemolysates of both fractionated intact cells and PH cells were prepared. The packed cells were suspended in 3 vol of hypotonic HEPES buffer (30 mOsm/kg, 5 mmol/L of HEPES, 10 mmol/L of NaCl; pH 7.4). After membrane components were removed by centrifugation (10,000 g for 20 minutes), the hemolysate was concentrated by ultrafiltration (using a collodion bag; SM13200, Sartorius-Membranefilter GmbH, Göttingen, FRG) until the hemoglobin concentration of the hemolysate reached that of the intracellular hemoglobin concentration of original cells. The viscosity of hemolysate of intact cells agreed with the reported value.²¹

RESULTS

Characterization of PH Cells

To facilitate the optical transparency of cell suspension and to avoid the decrease of internal viscosity, PH cells were prepared in the hypotonic dextran solution. Intact cells

resisted hypotonic hemolysis in the presence of dextran. Thus, to enhance hemolysis, cells were pretreated with LPC. By suspending LPC-treated cells in the hypotonic solution of different concentrations of dextran, the internal viscosity of PH cells could be varied. After resealing, LPC in the cell membrane was removed by washing with BSA solution and the cell shape could be easily reverted to biconcave disc. Although progressive loss of membrane materials caused by increasing LPC concentration has been reported,²² at a low concentration such as was used in the present study, no loss of cholesterol and phospholipids of either LPC-treated cells or PH cells was detected.

In this study, two kinds of PH cells, heavy and light, were prepared. The typical data about the properties of these cells are summarized in Table 1, together with data about density-fractionated intact cells for comparison. PH cells deformed to the flat ellipsoid with unequal axial lengths in shear flow as well as intact cells did, as reported by Suda and co-workers.²⁰ Thus, the degrees of deformation are expressed by DI in Table 1.

Deformation of Cells in Diluted Suspension

To investigate the influence of viscosity of suspending medium and shear rate on the degree of deformation, the DIs of cells suspended in the isotonic solution of various concentrations of dextran (hematocrit, 0.4%) were measured at various shear rates. Fischer and colleagues³ have shown that the DI of intact cells is a function of $\dot{\gamma}\eta^{1.5\pm0.2}$, where $\dot{\gamma}$ and η are shear rate and medium viscosity, respectively. In the present study, taking the intracellular viscosity into account, the variable $\dot{\gamma}\eta^{1.5\pm0.2}$ is modified to $\dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^\alpha$, where η_{ex} is the viscosity of the suspending medium, η_{in} is the intracellular viscosity, and α is the adjustable parameter.

PH cells. The DIs of light PH cells, at various shear rates and viscosities of suspending medium, are shown in Fig 1A. At certain DIs, the plots of $\log \dot{\gamma}\eta_{ex} \nu \log (\eta_{ex}/\eta_{in})$ shown in Fig 1B give parallel straight lines with the same slope (-0.6), where interpolated $\dot{\gamma}$ values were read at the crossing points on lines a through d of Fig 1. Thus, DI is expressed as a function of $\dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^{0.6}$. In Fig 2A, all DIs of light PH cells and heavy PH cells are plotted against $\dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^{0.6}$, and all points lie on the same curve in spite of the difference in η_{in} between light and heavy PH cells.

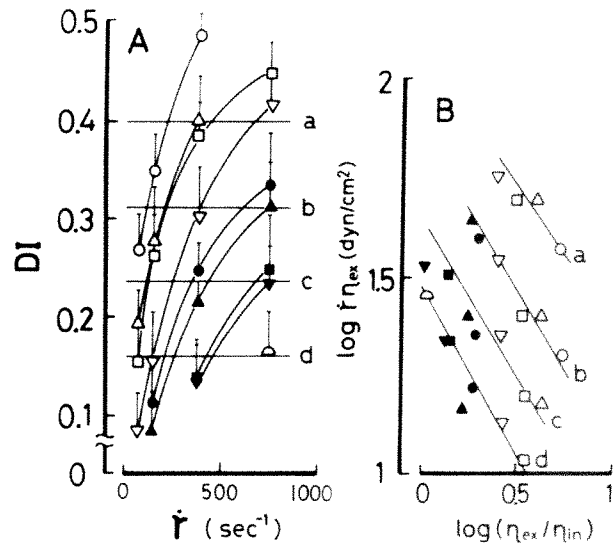


Fig 1. (A) Dependence of deformation index (DI) of light partially hemolyzed (PH) cells on the shear rate in the isotonic dextran solutions. Dextran concentration: 7.5% (Δ , 4.0 cP), 8.6% (∇ , 4.7 cP), 10% ($\blacksquare$, 5.1 cP), 11% ($\blacktriangle$, 6.1 cP), 12% ($\bullet$, 6.7 cP), 14% (∇ , 8.8 cP), 16% ($\square$, 11.5 cP), 18% (Δ , 14.5 cP), and 20% ($\circ$, 18.3 cP). DI is represented by mean $\pm$ SD of 60 to 80 cells. Lines a through d represent DI = 0.40, 0.31, 0.23, and 0.16, respectively; $\dot{\gamma}$ values at each DI value were estimated. (B) Logarithmic plot of shear stress ($\dot{\gamma}\eta_{ex}$) ν (suspending medium viscosity/intracellular viscosity) (η_{ex}/η_{in}). Data and symbols are the same as in A. The points on line a give a DI value of 0.40, on line b a DI value of 0.31, on line c a DI value of 0.23, and on line d a DI value of 0.16. The lines are drawn by linear regression analysis (mean $\pm$ SD of these slope = -0.60 ± 0.03).

Intact cells. Intracellular viscosity is well known to increase with cell aging. Therefore, intact cells were fractionated with Percoll to minimize the variation of intracellular viscosity. The same value of α was obtained for both low-density cells and high-density cells as that for PH cells in the plot $\log \dot{\gamma}\eta_{ex} \nu \log (\eta_{ex}/\eta_{in})$ (not shown). The curve of DI $\nu \dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^{0.6}$ of intact cells slightly shifts upward (Fig 2B) as compared with that of PH cells (dotted line is the curve for PH cells shown in Fig 2A). To fit these curves on the same line, another parameter β must be introduced; DI is then expressed as a function of $\beta\dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^{0.6}$. Adopting $\beta = 1$ for the intact cells, β for PH cells should be reduced to 0.7.

Table 1. Hematological Indices and Deformability of PH Cells and Fractionated Intact Cells

Cells	MCV (μm^3)	[Hb] _{in} * (mmol/L Cell)	$\eta_{in}\dagger$ (cP)	DI $\ddagger$	
				$\dot{\gamma} = 150 \text{ s}^{-1}$	$\dot{\gamma} = 376 \text{ s}^{-1}$
PH					
Light	96.0	2.58	3.5 (2.8)	0.35 ± 0.05	0.47 ± 0.02
Heavy	75.2	3.73	9.8 (5.0)	0.27 ± 0.05	0.41 ± 0.04
Intact§					
Low density	89.0	5.05	10.3	0.31 ± 0.04	0.44 ± 0.05
High density	81.5	6.20	20.4	0.22 ± 0.05	0.36 ± 0.05

PH, partially hemolyzed; MCV, mean corpuscular volume.

*Intracellular hemoglobin concentration.

$\dagger$ Intracellular viscosity. The value in parentheses is the viscosity of hemolysate (prepared from intact cells) having the same hemoglobin concentration as that of PH cells.

$\ddagger$ Deformation index (DI) was measured at shear rates ($\dot{\gamma}$) of 150 and 376 s⁻¹ when cells were suspended in 20% dextran solution (18.3 cP).

§Intact cells were fractionated by density gradient (described in Materials and Methods section).

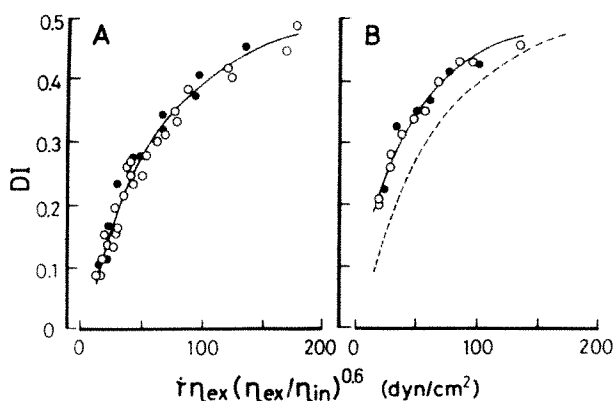


Fig 2. (A) Plot of deformation index (DI) $\nu \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$ of partially hemolyzed (PH) cells. Light PH cells (○) were suspended in isotonic dextran solutions of 7.5% (4.0 cP) to 20% (18.3 cP), and heavy PH cells (●) were suspended in solutions of 11% (6.1 cP) to 20%. DI was measured at shear rates of 75.2, 150, 376, 752 s⁻¹. Data of light PH cells are the same as in Fig 1A. DI is represented by a mean of 60 to 80 cells. (B) Plot of DI $\nu \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$ of intact cells. Fractionated low-density cells (○) were suspended in isotonic dextran solutions of 14% (8.8 cP) to 20% (18.3 cP), and fractionated high-density cells (●) were suspended in solutions of 16% (10.8 cP) to 20%. DI was measured at the same shear rates as in Fig 2A. The dotted line is the regression curve for PH cells shown in Fig 2A. DI is represented by a mean of 60 to 80 cells.

This fact implies that PH cells are less deformable than intact cells; presumably, the PH cell membrane would be impaired during preparation of PH cells.

Diamide-treated cells. Diamide has been shown to induce cross-linking of membrane proteins to decrease cellular deformability.^{15,23} When a high concentration of diamide is applied, a buckling pattern (furrows parallel to the flow direction) appears²⁴ and, in addition, hemoglobin is modified.¹⁵ Therefore, to study the influence of cross-linking of membrane proteins (mainly spectrin) on deformation, the concentration of diamide must be lowered to <1 mmol/L (no buckling pattern was detected).

DIs of diamide-treated cells were measured in the dextran solution from 15.5% (11.0 cP) to 20% (18.3 cP). The parameter α for diamide-treated cells was dependent on the degree of cross-linking, as estimated from the plot of $\log \dot{\gamma} \eta_{ex} \nu \log (\eta_{ex}/\eta_{in})$ (not shown). In Fig 3A, DIs are plotted against $\beta \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^\alpha$, with appropriate α values for cells treated with diamide of various concentrations whereas β is fixed at unity. Furthermore, adopting an appropriate value of β for each curve shown in Table 2, all points can be brought on the same curve (Fig 3B), which curve agrees with the curve for intact cells (Fig 2B), ie, both α and β decrease as the cross-linking proceeds.

Deformation of PH Cells Suspended in 6% Dextran Solution at Various Hts

Light PH cells. The rheoscopic flash photographs of light PH cells in relatively low Ht range (4% to 25%) at $\dot{\gamma} = 752$ s⁻¹ are shown in Fig 4 (line B). The ellipsoidal deformation becomes clear as hematocrit increases. This monotonous

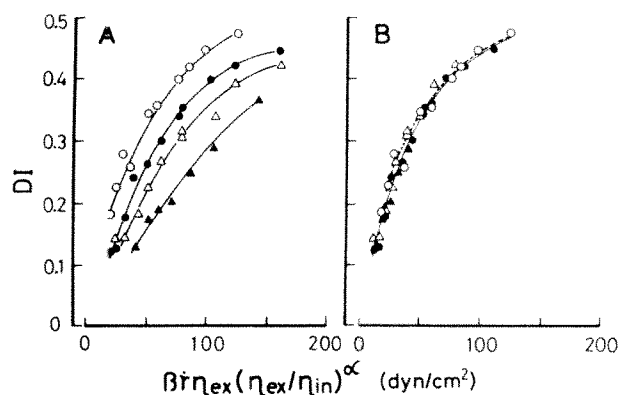


Fig 3. Plot of deformation index (DI) $\nu \beta \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^\alpha$ for diamide-treated cells. (A) The parameter α is varied whereas β is fixed at unity. Fractionated low-density cells were treated with diamide. Diamide concentration: 0 mmol/L (○), 0.55 mmol/L (●), 0.65 mmol/L (△), 0.95 mmol/L (▲). Cells were suspended in isotonic dextran solutions of 15.5% (11.0 cP) to 20% (18.3 cP), and DI was measured at shear rates of 75.2, 150, 376, and 752 s⁻¹; α values for cells treated with 0, 0.55, 0.65, and 0.95 mmol/L of diamide are 0.6, 0.5, 0.35, and 0, respectively. DI is represented by a mean of 60 to 80 cells. (B) Both parameters α and β are altered. Data and symbols are the same as in Fig 3A; β values for cells treated with 0, 0.55, 0.65, and 0.95 mmol/L of diamide are 1, 0.7, 0.5, and 0.4, respectively (α values are the same as in Fig 3A). The dotted line is the regression curve of DI $\nu \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$ of intact cells shown in Fig 2B. The degrees of cross-linking of spectrin of cells treated with different concentrations of diamide are shown in Table 2.

increase of deformation is due to increased suspension viscosity, from 3.4 cP (Ht = 4%) to 4.2 cP (Ht = 25%). When DIs are plotted against $\dot{\gamma} \eta_{sp} (\eta_{sp}/\eta_{in})^{0.6}$ (Fig 5), where η_{sp} is the suspensin viscosity, DIs lie on the DI $\nu \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$ curve of light PH cells shown in Fig 2A. Therefore, the increase of suspensin viscosity acts on cellular deformation in the same manner as the increase of viscosity of the dextran solution.

On the other hand, at higher Hts (>25%), DI measurement was impossible due to cell crowding. At a Ht of 45%, however, cells were photographically observed to deform to flat ellipsoid but variously distorted shapes and orient with their long axes parallel to the flow direction (Fig 4, line B).

Heavy PH cells. The ellipsoidal deformation could not be observed for heavy PH cells. Presumably, the value of $\dot{\gamma} \eta_{sp} (\eta_{sp}/\eta_{in})^{0.6}$ was not high enough to induce such deformation (eg, $\dot{\gamma} \eta_{sp} (\eta_{sp}/\eta_{in})^{0.6} = 19$ dynes/cm² at a Ht of 20%) due to the higher intracellular viscosity of heavy PH cells as compared with that of light PH cells.

Table 2. α and β Values of Diamide-Treated Cells

Diamide* (mmol/L)	Cross-linking† (%)	α	β
0	0	0.6	1
0.55	27.4	0.5	0.7
0.65	33.3	0.35	0.5
0.95	46.7	0	0.4

*Diamide concentration for diamide treatment of intact cells.

†Percentage of spectrin polymerization (described in Materials and Methods section).

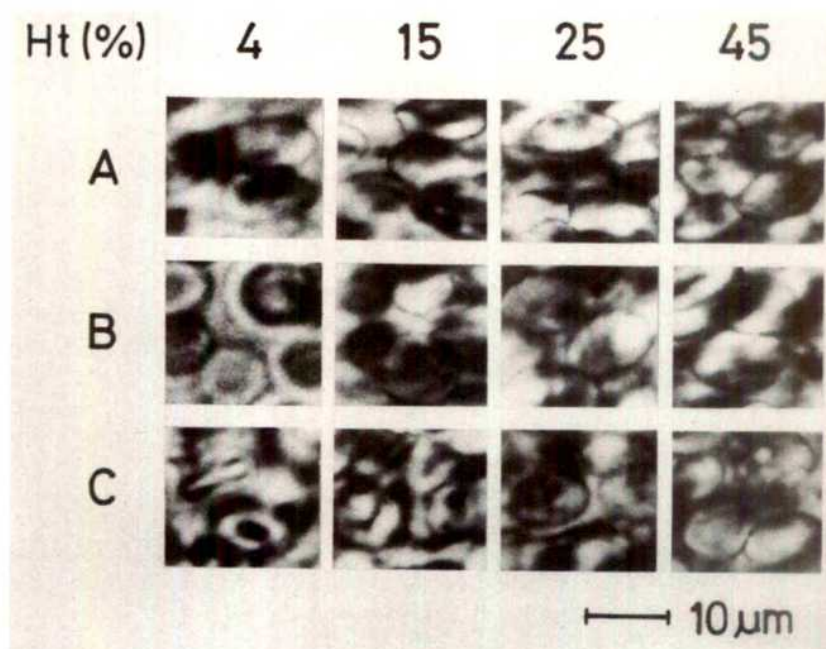


Fig 4. Deformation of light partially hemolyzed (PH) cells in the suspension of various hematocrits. Cells were suspended in 10% dextran solution (5.1 cP, line A), 6% dextran solution (3.3 cP, line B), and plasma (1.8 cP, line C). Cell deformation was observed at a shear rate of 752 s^{-1} . Hematocrit (Ht) of the suspension ranges from 4% to 45%.

Deformation of PH Cells Suspended in 10% Dextran Solution at Various Hts

Light PH cells. In 10% dextran solution, light PH cells demonstrate advanced ellipsoidal deformation as compared with those in 6% dextran solution at lower Hts (<25%) as shown in Fig 4 (line A). When DIs are plotted against $\dot{\gamma}\eta_{sp}(\eta_{sp}/\eta_{in})^{0.6}$, however, DI decreases from 0.28 to 0.23

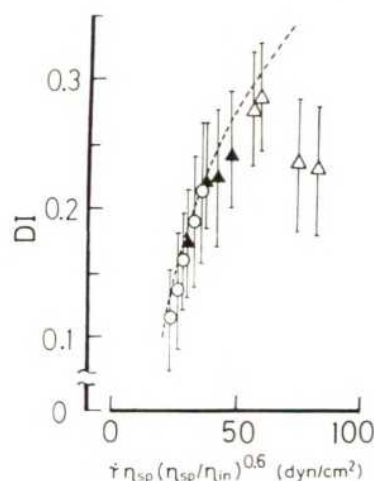


Fig 5. Effect of increasing hematocrit of partially hemolyzed (PH) cell suspension on deformation index (DI). Light PH cells were suspended in isotonic dextran solutions of 6% (3.3 cP, $\circ$) and 10% (5.1 cP, Δ). Heavy PH cells were suspended in 10% dextran solution ($\blacktriangle$). Deformation index (DI) was measured at a shear rate of 752 s^{-1} . Hematocrits of the suspension of light PH cells in 6% dextran solution were 4%, 8%, 15%, 20%, and 25%. Those of light PH cells in 10% dextran solution and heavy PH cells in 10% dextran solution were 4%, 8%, 15%, and 20%. DI is given as mean $\pm$ SD ($n = 60$ to 80). The dotted line is the regression curve of $\text{DI} \propto \dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^{0.6}$ of PH cells shown in Fig 2A.

despite the increase in $\dot{\gamma}\eta_{sp}(\eta_{sp}/\eta_{in})^{0.6}$ from 60 dynes/cm² (at Ht = 4%, $\eta_{sp} = 5.6$ cP) to 80 dynes/cm² (at Ht = 20%, $\eta_{sp} = 7.1$ cP) as shown in Fig 5. In Fig 4 (line A), a photograph at Ht = 45% is also shown, in which distorted ellipsoidal deformation is observed.

Heavy PH cells. Although the intracellular viscosity was high, the ellipsoidal deformation was induced due to high viscosity of the suspending medium. In the Ht range of 4% to 20%, as shown in Fig 5, DIs lie on the $\text{DI} \propto \dot{\gamma}\eta_{ex}(\eta_{ex}/\eta_{in})^{0.6}$ curve of light PH cells shown in Fig 2A. The suspension viscosity changes from 5.9 cP (Ht = 4%) to 7.6 cP (Ht = 20%).

Deformation of PH Cells Suspended in Plasma at Various Hts

Light PH cells. Cells deform to variously distorted discs at the lower Ht (<25%); however, distorted ellipsoidal deformation is conspicuous at Ht = 45%, as shown in Fig 4 (line C), at which cells deform to the same shape as that of light PH cells in 6% and 10% dextran solutions.

Heavy PH cells. Although the extracellular viscosity was low (1.8 cP), cells deformed but distorted in various shapes at the higher Ht range (35%, 45%, and 55%).

Deformation of Intact Cells in the Coexistence of Undeformable Cells

To investigate the influence of undeformable cells on the behavior of deformable cells in shear flow, a small amount of fractionated low-density intact cells (tracer cells) was mixed with either a larger amount of light PH cells (deformable cells) or GA-treated PH cells (light PH cells treated with GA, undeformable cells), and deformation of tracer cells was observed. GA-treated PH cells could not deform, and they oriented randomly in shear flow. Cells were suspended in

16.7% dextran solution (12.7 cP) in which the Ht of tracer cells was kept constant (0.4%), and the Ht of light PH cell and GA-treated PH cell suspensions varied from 2.1% to 6.3%. On the flash-photographs, the intact tracer cells could be distinguished from the light PH or GA-treated PH cells by differences of cell shape and of darkness (due to higher hemoglobin concentration). The ellipsoidal deformation of intact cells was not affected in the deformable light PH cell suspension (Fig 6B,C,D), nor in the diluted GA-treated PH cell suspension (Ht = 2.1%) (Fig 6E). When the amount of the GA-treated PH cells was increased, however (to Ht = 6.3%), the ellipsoidal deformation was remarkably disturbed (Fig 6F,G).

DISCUSSION

Contribution of Intracellular Viscosity and Membrane Stiffness to Cellular Deformation

Increased viscosity of suspending medium is well known to enhance the deformation of RBCs, although the degree of deformation is influenced by intracellular viscosity. Pfafferoth and co-workers²⁵ showed that the differences between young and old cell deformation were eliminated if the DI was plotted as a function of the ratio of intracellular/extracellular viscosities for various shear stresses.

In the present study, we demonstrated the effect of extracellular/intracellular viscosities and membrane stiffness on the cellular deformation and revealed that DI is a function of $\beta \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^\alpha$. For various kinds of cells, $\alpha = 0.6$ is unanimously applicable (Fig 2A,B). Taking $\beta = 1$ for the intact cells, β should be reduced to 0.7 for PH cells to fit the data of PH cells on the same curve, $DI \propto \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$, of intact cells, indicating that PH cells are less deformable than intact cells. A remarkable difference of cell volume between light and heavy PH cells is detected (Table 1), whereas the surface area of these cells remains constant (because no loss of membrane lipids is detected). This may affect the cellular deformation through the change of surface/volume (S/V) ratio which is one of the determinants of deformability. No difference, however, in the relation $DI \propto \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$

between light and heavy PH cells (Fig 2A) suggests that such change of S/V ratio does not shift the curves $DI \propto \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$ throughout. Therefore, the decrease in β of PH cells is presumably due to the increased membrane stiffness caused by changes in membrane integrity.

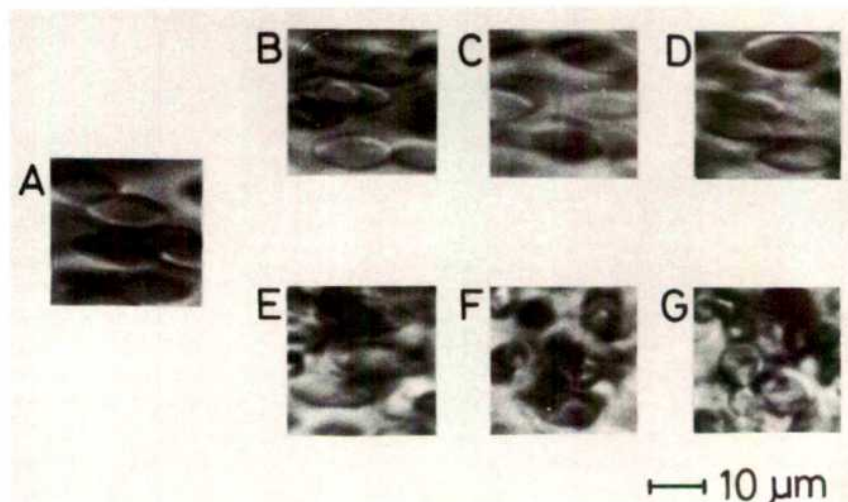
Although the intracellular viscosity of light PH cells is about half that of fractionated low-density cells, DIs of light PH cells are close to that of fractionated low-density cells at given shear stresses (Table 1), because the effect of lowered intracellular viscosity of PH cells on DIs is canceled by the increased membrane stiffness.

Age-related changes in membrane viscoelastic properties were reported.^{13,14,26,27} In this study, no difference in the $DI \propto \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^{0.6}$ curve between low-density cells and high-density cells was detected (Fig 2B). This result may imply that the age-dependent changes in membrane properties have no significant effect on the ellipsoidal deformation in shear flow.

The influence of cross-linking of membrane proteins is remarkable. When the intact cells (of low density) are treated with diamide, two parameters, α and β , must be reduced (Table 2) to bring all data to the same curve in the plot of $DI \propto \beta \dot{\gamma} \eta_{ex} (\eta_{ex}/\eta_{in})^\alpha$ (as shown in Fig 3).

The mechanical property of the membrane is physically expressed by the three intrinsic material constants of membrane, ie, elastic modulus, area compressibility, and membrane viscosity.^{28,29} The alteration of area compressibility would not be detectable in the present method, because discocytes deform easily at constant area but strongly resist change in area.³⁰ Nash and Meiselman demonstrated that the ghost has a greater shear modulus but a similar membrane viscosity.³¹ Their result may correspond to the present result, ie, the partial hemolysis causes the change in β but not in α . The parameter β may thus be related to the shear modulus. According to dimensional analysis, β should have a dimension of $(\text{dynes/cm}^2)^{-1}$. Therefore, a reciprocal of β would express the relative shear modulus, and the shear modulus of PH cells may be ~ 1.4 times higher than that of intact cells. The diamide treatment is also known to increase the shear modulus²⁴ and may cause increased membrane

Fig 6. Deformation of intact cell (fractionated low-density cell) in mixed suspension. Fractionated low-density cells (tracer cells) were suspended in 16.7% dextran solution (12.7 cP; the hematocrit of the tracer cells was adjusted to 0.4%) with either light partially hemolyzed (PH) cells or glutaraldehyde (GA)-treated PH cells. The deformation of tracer cells was observed at a shear rate of 752 s^{-1} . (A) Only tracer cells present in the suspension. (B through D) Tracer cells are mixed with light PH cells. Hematocrits of light PH cells in the suspensions of B, C, and D, are 2.1%, 4.3%, and 6.3%, respectively. (E through G) Tracer cells are mixed with GA-treated PH cells. Hematocrits of GA-treated PH cells in the suspensions of E, F, and G are 2.1%, 4.3%, and 6.3%, respectively.



viscosity³² by restricting the flexibility of cytoskeleton. This may lead to the decrease of two parameters, α and β .

Ht Dependency of Cellular Deformation

The shear rate applied in this study ($\dot{\gamma} = 752 \text{ s}^{-1}$) corresponds to the calculated wall shear rates in artery and capillary ($\sim 1,000 \text{ s}^{-1}$), assuming that Poiseuille flow³³ and the behavior of PH cells in flow is similar to that of intact cells. Therefore, the present study gives information on RBC behavior in blood flow.

The quantitative measurement of PH cell deformation is possible up to the Ht value of $\sim 20\%$. When the light and heavy PH cells are suspended in 6% and 10% dextran solutions, respectively, progressive deformation due to increase of suspension viscosity is observed. The increase in suspension viscosity owing either to increasing Ht or dextran concentration induces the same degree of ellipsoidal deformation (Fig 5). However, the value of $\dot{\gamma}\eta_{sp}(\eta_{sp}/\eta_{in})^{0.6}$ is below $\sim 20 \text{ dynes/cm}^2$, both light and heavy PH cells suspended in any medium cannot deform, because at low value of $\dot{\gamma}\eta_{sp}(\eta_{sp}/\eta_{in})^{0.6}$, the shear force is not enough to induce the deformation. On the other hand, DI of light PH cells suspended in 10% dextran at $\dot{\gamma}\eta_{sp}(\eta_{sp}/\eta_{in})^{0.6} = 80 \text{ dynes/cm}^2$ is smaller than that at $\dot{\gamma}\eta_{sp}(\eta_{sp}/\eta_{in})^{0.6} = 60 \text{ dynes/cm}^2$ as shown in Fig 5. In this decline phase, the ellipsoidal deformation is rather suppressed, probably because the cell crowding disturbs the uniform stream lines. Concerning the effect of Ht, Kon and Kon³⁴ have shown that the degree of deformation and orientation is suppressed as the Ht is increased above a certain value depending on extracellular viscosity; ie, the critical Ht value, at which the suppressing effect begins, shifts toward a lower Ht as the extracellular medium increases in viscosity. At higher Hts ($>25\%$), quantitative measurement of DI is not possible, even with PH cells. The degree of deformation of PH cells in plasma is qualitatively the same as that in 6% and 10% dextran solutions at a Ht of 45%, however. The ellipsoidal deformation of intact cells at a higher Ht is shown by Fischer and Schmid-Schönbein.² The

present result is explained as follows: The cellular deformation of PH cells in plasma is progressively induced with increasing Ht, but in 6% and 10% dextran solutions the suppressive mechanism becomes operative on cells at the higher Ht.

The influence of cell concentration was described by Goldsmith and Marlow³⁵; ie, the progressive deformation of tracer amount of intact cells in the ghost cell suspension (intact cells did not deform to ellipsoid but to irregularly distorted ellipsoid) with increasing ghost concentration; thus, they have proposed that the collision between cells induces such irregular deformation. In the present study, the fractionated low-density cells deformed to ellipsoid in light PH cell suspension but not in GA-treated PH cell suspension. This phenomenon may be due to the disturbance of laminar flow caused by tumbling of the hardened cells, which consequently reduces the effective shear stress acting on intact cells. Tumbling of rigid discs in Poiseuille flow is known,⁴ and a similar disturbing effect of hardened cells on intact cell deformation was reported by Kon and coworkers.³⁶ Therefore, the ellipsoidal deformation of intact cells is maintained by ellipsoidal deformation of surrounding cells.

In short, the present study reveals that: (a) The degree of deformation of cells is determined by the applied shear stress ($\dot{\gamma}\eta_{ex}$), the viscoelastic membrane properties (reflected on the parameters α and β), and the ratio of suspending medium viscosity to internal viscosity (η_{ex}/η_{in}); and (b) increasing suspension viscosity (η_{sp}) by raising Ht generally enhances cellular deformation, but above a certain Ht deformation is suppressed. At physiological Ht, cellular deformation is mainly influenced by Ht, and the contribution of viscosity of extracellular medium is not significant.

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Lack of Association Between Abnormalities of the Chromosome 9 Short Arm and Either "Lymphomatous" Features or T Cell Phenotype in Childhood Acute Lymphocytic Leukemia

By Andrew J. Carroll, Robert P. Castleberry, and William M. Crist

In childhood acute lymphocytic leukemia (ALL), abnormalities in the short (p) arm of chromosome 9, particularly those leading to the loss of material in the p21-p22 region, may be associated with bulky disease at diagnosis (so-called "lymphomatous" ALL) and a T cell immunophenotype. To assess these associations further, we reviewed the clinical and laboratory data for 100 consecutively evaluated children with ALL who had successful cytogenetic studies. From analysis of clinical and laboratory features, 8 of the 100 patients were classified as having lymphomatous ALL. Seven of the 100 patients had aberra-

tions involving the 9p arm or were missing an entire chromosome 9. The prevalence rate of 9p abnormalities in cases of lymphomatous ALL was not significantly different from that in cases without lymphomatous features (1 of 8 v 6 of 92, $P = .62$). Moreover, all seven patients with 9p abnormalities had the common ALL phenotype. These data suggest that although 9p abnormalities in childhood ALL occur frequently, there is no consistent association with either the occurrence of lymphomatous clinical features or the presence of T cell disease.

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THE CHROMOSOME complement of the leukemic clone is now recognized as an important prognostic variable in childhood acute lymphocytic leukemia (ALL).¹ Several clinically and/or immunologically defined subgroups of ALL have been associated with nonrandom cytogenetic abnormalities, including translocations such as t(4;11) in undifferentiated acute leukemia, t(8;14) in B cell ALL, t(1;19) in pre-B cell ALL,^{2,3} and t(11;14) in T cell ALL.³ Each of these is associated with a poor prognosis.

Kowalczyk and Sandberg⁴ identified a small, potentially important subgroup of patients with ALL, characterized by loss of material from the short arm of chromosome 9 (9p-), who typically were diagnosed with lymphadenopathy, splenomegaly, high leukocyte counts, and T cell markers on the surface of their leukemic blasts. More recently, Chilcote and colleagues⁵ proposed that loss of chromosomal material at 9p21-22 might be characteristic of patients with "lymphomatous" ALL. These children also had bulky extramedullary leukemia, often a T cell immunophenotype, and responded poorly to treatment. We therefore analyzed the clinical data for 100 consecutively evaluated children with ALL who had successful cytogenetic studies. We found no evidence to support the proposed association between 9p abnormalities and either lymphomatous clinical features or T cell disease.

MATERIALS AND METHODS

Patients. The study population was derived from children with ALL diagnosed and treated at The Children's Hospital of Alabama from 1978 through 1985. Informed consent, in accordance with institutional and FDA guidelines, was obtained prior to bone marrow aspiration. Clinical data from 100 consecutive patients with successful cytogenetic studies were analyzed. Lymphomatous ALL was defined on the basis of at least one clinical and one laboratory feature from the following list: discrete lymphadenopathy with nodes >3 cm in diameter or matted nodes >5 cm collectively; splenomegaly below the umbilicus; a mediastinal mass equal to more than one-third of the thoracic diameter; hemoglobin concentration >10 g/dl; leukocyte count >50,000/ μ l and E rosette positivity (E+) >25%. Patients were treated according to protocols of the Pediatric Oncology Group.

Immunologic characterization. After separation on a Ficoll-Hypaque gradient, leukemic marrow cells were evaluated by standard techniques for surface immunoglobulins (sIg),⁶ cytoplasmic immunoglobulins (cIg),⁷ and receptors for sheep erythrocytes.⁸ In

pre-B or B cell ALL, >10% of blasts expressed cIgs or sIgs, respectively. A case was considered E rosette positive if >40% of blasts formed heat stable E rosettes. The common ALL antigen (CALLA) was identified by microcytotoxicity with heteroantisera as previously described.⁹ CALLA positivity was present if blasts exhibited >40% lysis above control cells.

Chromosome analysis. Cells from bone marrow aspirates were processed directly and/or were subjected to short-term culture. For direct chromosome preparations, a fresh bone marrow aspirate (≤ 0.5 mL) was immediately placed into a sterile centrifuge tube containing 10 mL of 0.075 mol/L of KCl and colcemid (0.06 μ g/mL) and allowed to incubate at room temperature for 30 minutes. For short term culture, a bone marrow aspirate (≤ 0.5 mL) was placed in 10 mL of RPMI 1640 supplemented with 10% fetal calf serum (FCS) for 24 hours at 37 °C and then exposed to colcemid (0.06 μ g/mL for 2.5 hours at 4 °C. Routine methods were used for culture harvest, slide preparation, and GTG-banding. Karyotypic designations were assigned according to the International System for Human Cytogenetic Nomenclature.¹⁰

RESULTS

Sixty-one of the 100 cases studied had an acquired, cytogenetically abnormal leukemic clone. Seven (cases 1 through 7, Tables 1 and 2) had aberrations involving the short arm of chromosome 9; unbalanced rearrangements resulting in the loss of 9p material were noted in five (cases 1 and 4 through 7). Case 3 had a balanced translocation involving 9p22, and case 2 was missing an entire 9 chromosome. None of the six translocations involving 9p were identical. The source of the material that was translocated to

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Table 1. Clinical and Laboratory Findings for 14 Patients With "Lymphomatous" ALL With or Without Chromosome 9 Abnormalities

Case No.	Age/Sex	Lymph Nodes (>3 cm)	Spleen Size*	Mediastinal Mass†	Hb (>10 g/dl)	Leukocyte Count ($>50 \times 10^3/\mu\text{L}$)	E Rosettes (>25%)	CALLA	slg	clg
Abnormal 9p without lymphomatous features										
1	14/F	—	—	—	—	—	—	+	—	—
2	7/M	—	—	—	—	—	—	+	—	—
3	8/M	—	—	—	—	—	—	+	—	—
4	16/M	—	—	—	+	—	—	+	—	—
5	7/M	—	—	—	—	—	—	+	—	—
6	4/M	+	—	—	—	—	—	+	—	—
Abnormal 9p with lymphomatous features										
7	8/M	—	+	—	+	—	—	+	—	—
Normal 9p with lymphomatous features										
8	13/M	+	—	—	—	+	—	+	—	+
9	13/M	—	+	—	+	—	—	+	+	—
10	13/F	+	—	+	+	+	+	—	—	—
11	7/F	—	—	+	—	+	+	‡	—	—
12	16/M	—	—	+	+	+	+	—	—	—
13	7/M	+	—	—	—	+	+	‡	—	—
14	12/F	+	—	—	+	—	+	—	—	—

ALL, acute lymphocytic leukemia, CALLA, common ALL antigen.

*Spleen tip palpable below the umbilicus.

†Greater than one-third the thoracic diameter.

‡Inconclusive.

9p could be identified in four cases and involved chromosomes 10, 12p, 12q, and 13.

Eight children were diagnosed with lymphomatous ALL (cases 7 through 14, Tables 1 and 2). Their average age was 11.5 years as compared with 6.7 years for patients without lymphomatous characteristics. Notable clinical features included a leukocyte count $>50,000/\mu\text{L}$ in 5 cases, E rosette positivity in 5, clinically significant lymphadenopathy in 4, mediastinal mass in 3, and massive splenomegaly in 2.

Immunophenotypes were pre-B cell (1); B cell (1); non-B, non-T, non-pre-B (1); and T cell (5). The proportion of T cell disease was predictably higher in the lymphomatous subset as compared with the remainder of the patient population (5 of 8, 63% v 6 of 92, 7%). The overall distribution of cases according to immunophenotype was non-B, non-T, non-pre-B (58); T cell (11); B cell (3); and pre-B cell (28).

Table 1 compares the clinical and laboratory findings for the 14 patients with lymphomatous ALL, with or without an

Table 2. Cytogenetic Findings for the 14 Patients With "Lymphomatous" ALL With or Without Chromosome 9p Abnormalities

Case No.	No. of Cells Examined	Abnormal Cells (%)	Karyotype
Abnormal 9p without lymphomatous features			
1	32	78	46,XX/58,XX,-9,-13,-18,+X,+2,+4,+5,+5,+6,+8,+8,+10,+10,+20,+21,+21,+22,+der(9),t(9;13)(p21;q13)
2	19	100	47,XY,-9,+12,+mar
3	21	100	46,XY,t(2;12)(p13;p12),t(9;10)(p22;q21)
4	22	45	46,XY/45,XY,-9,-12,+der(9),t(9;12)(p11;q11)
5	20	95	46,XY/60,XY,-15,+X,+2,+4,+5,+8,+10,+11,+12,+14,+18,+19,+20,+21,+21,+del(6)(q21→qter),der(9),t(9;?) (p13;?)
6	21	86	46,XY/45,XY,-1,der(6),t(6;?) (p25;?),del(7)(q22→qter),der(9),t(9;?) (p13;?)
Abnormal 9p with lymphomatous features			
7	21	95	46,XY/45,XY,del(7)(p15→pter),t(9;12)(p11;p13)
Normal 9p with lymphomatous features			
8	24	96	46,XY/46,XY,t(9;22)(q34;q11),t(8;14)(q11;q32)(88%)/47,XY,t(9;22)(q34;q11),t(8;14)(q11;q32),+Ph ¹ (8%)
9	20	0	46,XY
10	19	79	46,XX/45,XX,-10,t(4;5)(q11;q35)(11%)/90,XXXX,-10,-10,t(4;5),t(4;5)(68%)
11	19	0	46,XX
12	20	0	46,XY
13	13	69	46,XY/46,XY,-10,+16,+21,t(13q14q)
14	18	17	46,XX/45,XX,-20,t(7;?) (p22;?),t(4;?) (p14;?)

abnormal chromosome 9p. All seven children with 9p abnormalities had the common ALL phenotype (CALLA⁺, E⁻, sIg⁻, cIg⁻). Only one child (case 7) had both lymphomatous ALL and an abnormal 9p chromosome. The prevalence rate of 9p abnormalities in cases of lymphomatous ALL was not significantly different from that in cases without these features (1 of 8 v 6 of 92, $P = .62$). In addition, lymphomatous features were as likely to be found in patients without 9p abnormalities as in those who had these features (7 of 93 v 1 of 7, $P = .63$).

All children with 9p abnormalities attained complete remission (CR). Only one (case 2) has relapsed in bone marrow (after 9 months of CR), and he is presently in CR 4 years after a bone marrow transplant. The remainder of this group continue in CR for 6 to 36 months (mean 16 months). Three of the eight patients with a lymphomatous presentation never attained CR. Of the 5 children achieving remission, 3 have relapsed in the bone marrow and 1 has relapsed in the central nervous system. The child who had both lymphomatous clinical features and a 9p abnormality (case 7) has been in continuous CR for 27 months.

DISCUSSION

This study demonstrated that abnormalities of the chromosome 9 short arm are nonrandom in childhood ALL, occurring in ~7% of affected children. No significant association between abnormalities of 9p and the T cell immunophenotype or the so-called lymphomatous presentation of ALL was observed. The overall incidence of T cell ALL in our population of children with ALL and satisfactory cytogenetic analyses was 11%, which is similar to that described in large series of children with ALL.¹¹ Furthermore, the 8% incidence of lymphomatous presentation noted in our series is similar to the 10% reported by others.^{12,13} All series reported have noted a poor outlook for children with lymphomatous presentations, as expected, since by definition such patients have more bulk disease and are generally older, factors that have adverse prognostic significance.

Although most of the patients studied by Chilcote and colleagues and by Kowalczyk and Sandberg had 9p deletions, most of our patients had translocations involving 9p. In all three series, the abnormalities centered around the 9p21-22 region. In most cases that involved a translocation, the rearrangement was unbalanced and resulted in the loss of 9p material. In addition, the observation that an abnormal chromosome 9p was involved with different donor chromosomes (8⁵, 10, 12p, 12q, 13, and 21⁴) suggests that deletion or rearrangement of material in the 9p21-22 region may play a role in malignant transformation of the cells.

Our observations regarding the lack of an association between abnormalities of the 9p and lymphomatous and/or T cell ALL are different from those of both Kowalczyk and Sandberg⁴ and Chilcote and colleagues.⁵ This difference could in part relate to the older age distribution of the patients of interest studied in the two comparable study populations with 9p chromosome abnormalities. Four of the 7 patients with the 9p in Chilcote's series were >16 years of age whereas only 2 of 7 of our patients were aged >14 years. The mean age in the series of Kowalczyk and Sandberg was 12.7 years. A known increased incidence of T cell ALL is associated with lymphomatous presentation in adolescent patients.¹⁴ Six out of seven of our children with ALL and the 9p were males, but only one of them was an adolescent (16 years). Thus, the older age distribution in other populations could result in more patients being classified as having lymphomatous leukemia. The selection of hemoglobin (Hb) >10 g/dL as a criteria for lymphomatous ALL may also skew the study population mean age since higher Hb at diagnosis have been associated with older age. A male predominance was also noted in both previous series. Moreover our series of 100 children is larger than that reported by Chilcote and colleagues (41 children) or by Kowalczyk and Sandberg (70), and our immunophenotype distribution is typical for childhood ALL. Our success rate (61%) in obtaining abnormal karyotypes compares favorably to the series by Kowalczyk and Sandberg (60%). The success rate for the series reported by Chilcote and colleagues was not mentioned. Because cytogenetic methodologies used in both comparable studies are similar to our method, we doubt that technical differences account for the difference in our findings.

Recently, other nonrandom translocations have been shown to be specific for certain clinical syndromes of acute or chronic leukemia, or for specific immunophenotypes of ALL.^{2,3,15} Furthermore, some of these translocations occur near genes encoding proteins involved with cell growth.¹⁶ In regard to abnormalities of 9p, the recent observation of Carrera and co-workers¹⁷ that the gene encoding methylthioadenosine phosphorylase, an enzyme important in purine and polyamine metabolism in proliferating lymphocytes, is located on chromosome 9 is of considerable interest. The precise role of each of these chromosomal translocations in the process of malignant transformation is presently being investigated intensely.

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Increased Resistance to Membrane Deformation of Shape-Transformed Human Red Blood Cells

By Anne Chabanel, Walter Reinhart, and Shu Chien

The study of shape-transformed human erythrocytes is of hematological interest since several clinical conditions are associated with erythrocyte shape changes. Using the micropipette technique, we examined the rheological behaviors of echinocytes induced by sodium salicylate or ATP depletion and of stomatocytes induced by chlorpromazine. Both the discocyte-echinocyte and discocyte-stomatocyte transformations resulted in an increased resistance

to extension. Sodium salicylate-treated erythrocytes could be returned to normal morphology and normal membrane rheology by addition of chlorpromazine. We conclude that these morphological changes are associated with detrimental effects on the intrinsic membrane deformability of the human erythrocyte.

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EVIDENCE SHOWS that shape changes of the erythrocyte found in clinical conditions or induced by experimental manipulation are associated with alterations in erythrocyte rheology, which may have detrimental influences on the microcirculation. Abnormal rheologic behavior of non-discocytic, pathologic erythrocytes have been described in several hematological disorders, including sickle cell disease,¹ hereditary elliptocytosis,² hereditary spherocytosis,³ and hereditary stomatocytosis.⁴ Alterations in erythrocyte shape can also be induced by a variety of chemical agents.⁵ Treatment of fresh human erythrocytes with lysolecithin, oleate,^{6,7} 2,4-dinitrophenol,⁷ sodium salicylate (SA),⁸ alkaline medium,⁶ or the Ca^{2+} -ionophore A23187,⁹ as well as ATP depletion,¹⁰ can lead to crenation, ie, formation of echinocytes. On the other hand, erythrocytes become cup-shaped, ie, stomatocytes, following treatment with chlorpromazine¹¹ or local anesthetics,⁸ as well as from cholesterol depletion.^{12,13} These shape changes occur rapidly and are reversible if the distortion is not carried out to the spherocyte stage. These shape transformations might be expected to cause alterations in the rheological properties of erythrocytes through direct modification of the cell geometry (surface area/volume ratio) or through associated alterations of the membrane skeleton. Indeed, several groups have reported that drug-induced shape changes of erythrocytes are accompanied by alterations in the rheological properties of erythrocyte suspensions.^{9,14-18} Reinhart and Chien¹⁸ found that erythrocyte passage through a narrow channel is impaired for stomatocytes and facilitated for echinocytes; these changes in rheological behavior were explained by altered surface area/volume relationship, as the isovolumic shape transformations were accompanied by a loss of surface area in stomatocytes and a gain of surface area in echinocytes. Because microsieving measurements cannot clearly dissociate the contributions of the various determinants of cell deformability (cell geometry, internal cell viscosity, and membrane mechanical properties), the present experiments were performed to investigate directly the membrane mechanical properties of shape-transformed erythrocytes by using the micropipette aspiration technique. Indeed, the micropipette test is suited to the study of membrane mechanical properties since it has been shown that the viscous dissipation inside the RBC in a small deformation during aspiration into a micropipette is negligible under isotonic conditions.¹⁹

MATERIALS AND METHODS

RBC suspensions. Blood was drawn from healthy human volunteers into heparinized vacutainer tubes. The blood was centrifuged at $1,500 \times g$ for 10 minutes, and the plasma and the buffy coat were removed. The erythrocytes were washed three times in a standard buffer used throughout this study; this buffer contained 0.85 g/dL of NaCl, 0.25 g/dL of bovine serum albumin (BSA) and 0.1 g/dL of EDTA; its pH was adjusted to 7.4 with 0.1 N Tris. The washed erythrocytes were resuspended in the buffer at a hematocrit of 10%.

Stomatocytes were produced in vitro with chlorpromazine hydrochloride (CP), and echinocytes were produced with SA. A calculated volume of the suspending medium of the 10% erythrocyte suspension was replaced by an equal volume of a stock solution of CP (1.5 mmol/L) or SA (160 mmol/L). The suspensions were incubated for 20 minutes at room temperature and then used for measurements. ATP-depleted cells were obtained by incubation of whole blood under sterile conditions for 24 hours at 37 °C. The depleted erythrocytes were resuspended in the autologous plasma to a hematocrit of 10%.

Micropipette technique. The micropipette technique has been described elsewhere.²⁰ Micropipettes with an internal radius (R_p) of 0.45 to 0.70 μm were prepared with a micropipette puller (Narishige Scientific Instrument Laboratory, Tokyo). The micropipette was filled with the buffer solution and mounted on a micromanipulator (Narishige). The wide end of the micropipette was connected to a pressure-regulating system, which consisted of two reservoir bottles and a damping chamber. By adjustment of the relative heights of the reservoir bottles with a micrometer device, desired pressure levels were preset and then imposed on the micropipette by turning a stopcock. The applied pressure was measured with a transducer (model 23 BC, Statham Instrument, Oxnard, CA) and recorded with an amplifier-recorder system (Gould, Cleveland). A suspension

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of erythrocytes at a hematocrit level of $\sim 0.05\%$ was placed in a small round chamber located on the stage of a Nikon inverted microscope (Ehrenreich Photo-Optical, Garden City, NY). The erythrocytes were viewed with the use of a $100\times$ objective and a $20\times$ eye piece. The image was recorded with a video camera and tape recorder system (Panasonic, Division of Matsushita Electric of America, Franklin Park, IL). The micropipette tip was manipulated for positioning at the surface of the erythrocyte membrane. A small portion of the erythrocyte was aspirated by a preset negative pressure for 20 seconds. The length of the aspirated tongue and the radius of the pipette were measured on a television screen. The membrane extensional rigidity, which reflects the steady-state resistance to deformation, was calculated from the relationship between the stress applied, $(\Delta P)R_p$, and the strain induced, D_{pm}/R_p ,²⁰ where ΔP is the applied negative pressure, R_p is the internal radius of the micropipette, and D_{pm} is the maximum length of the aspirated portion within the micropipette. When the aspiration pressure was removed, the deformed erythrocyte segment in the micropipette decreased in length with time, and the cell recovered its original shape. All measurements were made at room temperature (21 to 24°C). For each experiment, the same micropipette was used to test shape-transformed RBCs and control RBCs. Statistical analysis of the data was performed by comparison of the slope of the linear regression using a paired Student's *t* test²¹ or by analysis of variance followed by the Newman-Keuls test.²²

Scanning electron microscopy. RBC specimens were fixed in 1.0% glutaraldehyde in cacodylate buffer. After two cacodylate buffer rinses, the specimens were processed through an ethanol dehydration series from 30% to 100% ethanol. The cells were resuspended in 100% ethanol and gently dropped onto a glass coverslip that had been cleaned with ethanol. After being air-dried, the coverslip with cells was glued onto aluminum stubs (JEOL USA, Electron Optics Division, Peabody, MA) with silver conductive paint. The stubs with samples were coated with gold-palladium in a sputterer (Hummer I; Technics, Alexandria, VA), and the specimens were viewed and photographed in a scanning electron microscope (JEOL 35; JEOL USA) at 25 kV .

RESULTS

Bessis' nomenclature²³ was used to classify erythrocytes according to their shape. Thus, a discocyte is a normal biconcave RBC with a smooth contour, echinocyte I is a flat erythrocyte with an irregular contour, echinocyte II is a flat erythrocyte with one or several spicules, and echinocyte III is an ovoid or spherical cell with numerous spicules evenly distributed over its surface. Stomatocytes are bowl-shaped cells with a single concavity (stoma); the concavity is large and shallow in stomatocytes I, deep and narrow like a token-slit in stomatocytes II, and deep and folded in stomatocytes III. Spherostomatocytes have a smaller diameter and small irregular invaginations. The morphology corresponding to this classification is illustrated in Fig 1.

Erythrocytes incubated with 25 or $100\text{ }\mu\text{mol/L}$ of CP produced stomatocytes I through III. Incubation of erythrocytes with 20 mmol/L of SA or depletion of their ATP content for 24 hours at 37°C produced echinocytes over the whole range from echinocytes I to spheroechinocytes. The micropipette test was carried out on every type of erythrocyte. Their mechanical behavior can be compared by plotting the steady-state deformation (D_{pm}) normalized for the pipette radius (R_p) against the stress parameter $(\Delta P)R_p$. When the aspiration was performed at a spicule site, the length of the protrusion inside the pipette prior to the application of

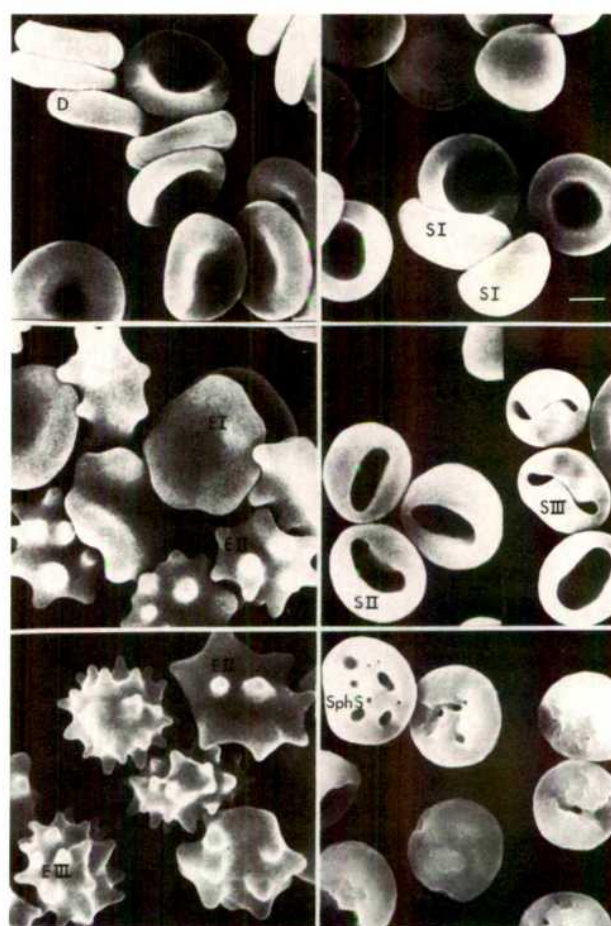


Fig 1. Scanning electron micrographs of control discocytes (D); echinocytes type I (EI), type II (EII), and type III (EIII); stomatocytes type I (SI), type II (SII), and type III (SIII); and spherostomatocytes (SphS). Bar = $2\text{ }\mu\text{m}$.

aspiration pressure was subtracted from the length of the tongue induced by the negative pressure to give the steady-state deformation (D_{pm}). Figure 2 shows the results for the echinocytes. The slope (*K*) of the stress-strain relationship was obtained by linear regression analysis of the data on D_{pm}/R_p v $(\Delta P)R_p$. The extensional rigidity was calculated as $1/0.245\text{ K}$; the value of 0.245 was based on the model described in ref. 20. Because the geometry at the point of aspiration is not an infinite plane, in shape-transformed RBCs we could not readily use the mathematical model to derive values for the intrinsic membrane elastic modulus; ie, due to the geometry of the cell, the micropipette test cannot distinguish between an effect on the membrane elastic modulus itself and a consequence in overall cell geometry that might generate local membrane tensions. By analogy with the work of Evans and colleagues²⁴ and Nash and co-workers,²⁵ who studied sickle cells (another type of abnormally shaped RBC) by micropipette test, we used values for the extensional rigidity to allow simple comparison of the resistance to deformation of shape-transformed RBCs and control discocytes. Extensional rigidity significantly and progressively increased with the degree of echinocytosis (Table I).

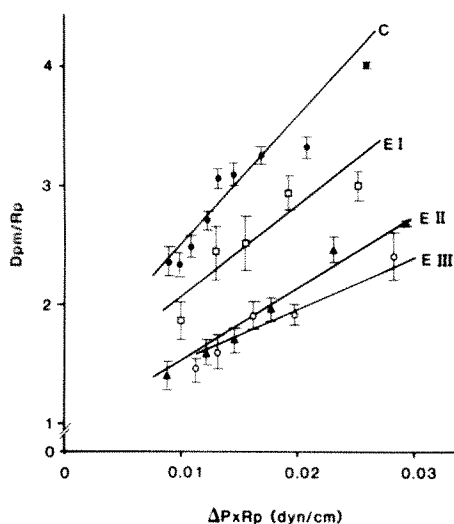


Fig 2. Strain (D_{pm}/R_p)–stress ($\Delta P \times R_p$) relationship for discocytes (C, ●); and echinocytes type I (EI, □), type II (EII, ▲), and type III (EIII, ○). Lines represent linear regressions computed from n data pairs for each group ($n > 55$). Data points were grouped according to $\Delta P \times R_p$, with mean $\pm$ SEM shown for each group.

There was no significant difference in membrane rheological behavior between the echinocytes induced by SA or ATP depletion (Table 1). Less stress was required to deform an echinocyte between the spicules than at a spicule site (Table 1). The intercept on the strain axis (ordinate) at zero stress decreased with the degree of echinocytosis (Fig 2).

Because it was often difficult under light microscopy to determine the degree of stomatocytosis, stomatocytes I through III were grouped together in the analysis. Figure 3 compares the elastic behavior of stomatocytes I through III and spherostomatocytes induced by different concentrations of CP to that of control discocytes. The value of extensional rigidity for the control cells in the stomatocyte set of experiments (Table 2) was different from that in the echinocyte set of experiments (Table 1). These two sets of experiments were performed on different days with different blood

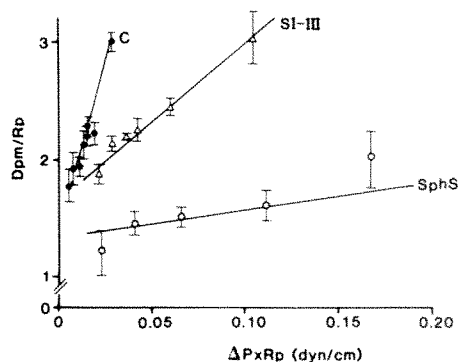


Fig 3. Strain (D_{pm}/R_p)–stress ($\Delta P \times R_p$) relationship for discocytes (C, ●), stomatocytes types I through III (SI through III, Δ), and spherostomatocytes (SphS, ○). Lines represent linear regressions computed from n data pairs for each group ($n > 39$). Data points were grouped according to $\Delta P \times R_p$, with mean $\pm$ SEM shown for each group.

donors. The overall range of values for extensional rigidity for the control cells was 3×10^{-3} to 12×10^{-3} dynes/cm. The stress needed to obtain a comparable D_{pm}/R_p was approximately three times greater for stomatocytes I through III than for discocytes, and it was ~ 30 times greater for spherostomatocytes than for discocytes (Fig 3). Values for the extensional rigidity are shown in Table 2.

These data show that both the discocyte–echinocyte and the discocyte–stomatocyte transformations resulted in increases of membrane extensional rigidity. The membrane rheological behavior of erythrocytes that had been transformed to echinocytes by SA (20 mmol/L) and subsequently returned to a discocytic shape by addition of CP (2.5 μ mol/L) was also studied (Fig 4). With the insertion of both of these drugs in the erythrocyte membrane, the rheological behavior of the membrane returned to normal as the discocytic shape was recovered.

DISCUSSION

This study demonstrates that membrane deformability is decreased in shape-transformed erythrocytes (both echinocytes and stomatocytes) as assessed by the micropipette technique. Although no data exist on stomatocytes, several groups have studied echinocytes. Leblond,⁶ using a micropipette with a diameter of 1.5 μ m, found a decreased membrane deformability in echinocytes induced by oleate, high pH, aged plasma, and ATP depletion, which is in agreement with our results. Baker,²⁶ measuring erythrocyte deformabil-

Table 1. Comparison of Membrane Elasticity Between Discocytes and Echinocytes

Group	No. of Cells Tested	Extensional Rigidity* (10^{-3} dynes/cm)	P Value
Discocytes	55	3.8 ± 0.2	
Echinocytes I	25	5.3 ± 0.6	NS†
Echinocytes II	55	6.8 ± 0.4	<0.05 †
Echinocytes III	53	9.6 ± 1.0	<0.05 †
Comparison of different sites on echinocytes II			
Between spicules	16	4.5 ± 0.3	
At spicules	55	6.8 ± 0.4	<0.05 ‡
Comparison of two methods for producing echinocytes III			
Salicylate	36	7.6 ± 0.7	
ATP depletion	17	8.9 ± 1.1	NS‡

*Values are mean $\pm$ SEM.

†P values are based on an analysis of variance followed by the Newman-Keuls test for the different types of echinocytes as compared with discocytes.²²

‡P values are based on Student's t test (two-sided).²¹

Table 2. Comparison of Membrane Elasticity Between Discocytes and Stomatocytes

Group	No. of Cells Tested	Extensional Rigidity* (10^{-3} dynes/cm)	P values†
Discocytes	50	7.5 ± 0.4	—
Stomatocytes I–III	94	29.8 ± 1.7	0.01
Spherostomatocytes	16	163.9 ± 20.4	0.01

*Values are mean $\pm$ SEM.

†P values are based on an analysis of variance followed by a Newman-Keuls test for the different types of stomatocytes as compared with discocytes.²²

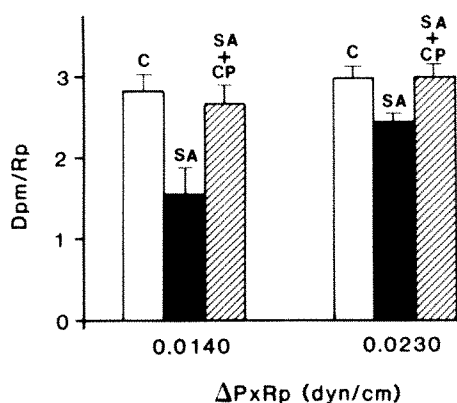


Fig 4. Normalized membrane deformation (D_{pm}/R_p) at two different deforming stresses ($\Delta P \times R_p$) for control discocytes (C), echinocytes produced by 20 mmol/L of salicylate (SA), and after biconcave shape recovery with the addition of 2.5 μ mol/L chlorpromazine (SA + CP). The vertical bars represent SEM.

ity by aspiration in Nuclepore filters with 0.6- μ m pores, found that ATP-depleted erythrocytes were 16% less deformable than fresh cells. Meiselman and co-workers,²⁷ however, using the micropipette test, reported that the decreased membrane deformability of ATP-depleted erythrocytes was not statistically significant. In these two last studies, the authors did not report any correction for the length of the spicule at rest inside the micropipette or the filter pore. The absence of such correction would lead to an overestimation of the length of the pressure-induced deformations and an underestimation of the elastic modulus for the echinocytes; this may explain the lesser effects seen in their results as compared with the present data. When we subtracted the mean spicule length from the pressure-induced pseudopod length, using Baker's technique with Nuclepore filters,²⁶ the membrane deformability of echinocytes also decreased (W. Reinhart, A. Chabanel, and S. Chien, unpublished observations, April 1985) to an extent similar to that found by the micropipette technique in the present study. Leblond⁶ reported that ATP-depleted cells were much more rigid than drug-crenated cells. Although we also observed a greater rigidity of the ATP-depleted echinocytes as compared with the echinocytes induced by SA, the difference was not statistically significant. Determining the deformability of resealed human erythrocyte ghosts in the ektacytometer, Shrier²⁸ found that the echinocytic ghosts were more deformable than the discocytic ghosts, whether the ghosts were prepared from discocytic or echinocytic erythrocytes. He concluded that acquisition of the discocytic shape in ghosts is accompanied by an increase in membrane rigidity. The reason for the discrepancy between his results and ours is not clear. One possible explanation is that the influence of the cellular geometry is dominant over that of membrane deformability in measurements performed with the ektacytometer. In that case, Schrier's results are consistent with the results of Reinhart and Chien¹⁸ on filtration through narrow pores, in which cell geometry is a major determinant of filterability; they found that echinocyte passage through narrow pores is facilitated rather than retarded

as compared with that of discocytes. Meiselman and co-workers reported that the suspension viscosity of echinocytes induced isovolumically by 2,4-dinitrophenol, 2,4,6-trinitrophenol, or SA¹⁷ and by ATP depletion¹⁵ was higher than that of normal cell suspensions at low shear rates but nearly normal at high shear rates. Because it was believed that change in cellular deformability would be reflected by a change in suspension viscosity at high shear rate, they concluded that altered morphology, rather than erythrocyte membrane deformability, was the cause of the rheologic changes. Minor or moderate changes in membrane rheology, however, may be detected by suspension viscosity measurements only at low or medium shear rates.²⁹ Considered thus the viscometric results obtained by Meiselman and Baker^{15,17} are in accord with our micropipette measurements in showing that shape transformation of erythrocytes results in an increased resistance to membrane extension.

This increase in resistance to membrane extension could be the consequence of (a) changes in the molecular organization of the membrane due to drug insertion or ATP depletion and/or (b) changes in the physical properties due to modification of the cell shape. Several groups have reported changes of the protein-lipid interface induced by CP,³⁰⁻³³ suggesting that interaction between the drugs (CP or SA) and the erythrocyte membrane constituents may lead to the increase in membrane rigidity. When both drugs were incorporated to cause the recovery of normal biconcave cell shape, we found that membrane deformability also returned to normal. This is in agreement with the results of Meiselman,¹⁷ who showed that both the morphology and rheology of 2,4-dinitrophenol-treated erythrocytes could be returned toward those of fresh control erythrocytes by addition of the antagonist chlorpromazine. Although we cannot rule out the possibility that when both drugs are added they have mutually compensating effects in their interaction with the membrane constituents, our data seem to support the concept that erythrocyte morphology per se has an important relation to the membrane mechanical properties. Leblond⁶ has proposed that the increased resistance to deformation observed in echinocytes may reflect the presence of new forces whose action is to produce foldings on the cell surface and hence to resist extension into the micropipette in response to pressure aspiration. We found that the intercept on the strain axis at zero stress decreased with the degree of echinocytosis (Fig 2), indicating that increasing stresses are required to reach a given extensional deformation. This might reflect an increase in the resting membrane tension as proposed by Leblond.⁶ Although we do not know if the spicule sites are predetermined, the present data show that the formation of spicules on an erythrocyte results in regional variations of physical properties on the membrane.

In conclusion, the present data demonstrate that changes in local membrane properties associated with overall erythrocyte shape transformation affect its membrane deformability. The decrease in erythrocyte membrane deformability following shape transformation could be of importance in the microcirculation when the erythrocyte must deform to negotiate its entrance into narrow capillaries.

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High-Dose Cytosine Arabinoside and Mitoxantrone: A Highly Effective Regimen in Refractory Acute Myeloid Leukemia

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In a clinical phase I/II study, high-dose cytosine arabinoside and mitoxantrone (HAM) were given in combination to 40 patients with refractory acute myeloid leukemia. All patients had received a 9-day combination of thioguanine, Ara-C, and daunorubicin (TAD-9) as standardized first-line treatment. Refractoriness was defined as (a) nonresponse against two TAD-9 induction cycles, (b) early relapse within the first 6 months on monthly maintenance or after TAD-9 consolidation, (c) relapse after 6 months with nonresponse against one additional TAD-9 cycle, and (d) second and subsequent relapses after successful TAD-9 therapy at the preceding relapse. Therapy consisted of HD-Ara-C 3 g/m² every 12 hours on days 1 through 4; mitoxantrone was started at 12 mg/m²/day on days 3, 4, and 5 and was escalated to 4 and 5 doses of 10 mg/m²/day on days 2 through 5 and 2 through 6. Of the 40 patients, 21 achieved a complete remission (53%), 1 patient had a

partial remission, and 5 patients were nonresponders. Thirteen patients died in aplasia due to infections (n = 11), pericardial effusion, or acute cardiomyopathy. Nonhematologic side effects consisted predominantly of nausea and vomiting, mucositis, and diarrhea. Central nervous system (CNS) symptoms were observed during six treatment courses. Recovery of blood counts occurred at a median of 27 days from the onset of treatment; the median time to complete remission was 36 days. Two of the 21 responders underwent successful bone marrow transplantations. The median remission duration for the remaining 19 patients is 4.5 months, and the median survival time is 9 months. These data emphasize that HAM has high antileukemic activity in refractory AML and strongly suggest starting the combination at earlier stages in AML therapy.

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DESPITE INTENSIVE induction therapy and subsequent myelosuppressive treatment in remission, most patients with acute myeloid leukemia (AML) still relapse with their disease and ultimately die of drug-resistant leukemia. High secondary remission rates and even prolonged survival have recently been reported, however, with administration of high-dose cytosine arabinoside (HD-Ara-C) alone or in combination with anthracyclines, asparaginase, or m-AMSA.¹⁻⁶ Patients with leukemias refractory to conventional-dose Ara-C especially benefitted from the addition of anthracyclines.⁵ These data are in accordance with previous cell kinetic studies revealing a conditioning effect of Ara-C for the subsequent administration of daunorubicin or doxorubicin.⁷⁻¹⁰ They are further supported by recent experimental results indicating a time-dependent synergistic effect of high-dose cytosine arabinoside (HD-Ara-C) and mitoxantrone.¹¹

These findings and the significant antileukemic activity of mitoxantrone as a single agent¹²⁻¹⁶ as well as its low cardiac toxicity¹⁷⁻²⁰ prompted us to initiate a phase I/II study of HD-Ara-C and mitoxantrone (HAM) in patients with refractory AML. All patients were recruited from the multi-

center German AML Cooperative Group trial^{21,22} and had therefore received a standardized first-line treatment with a 9-day combination of thioguanine, Ara-C, and daunorubicin (TAD-9).

HD-Ara-C was given every 12 hours at a dose of 3 g/m² by a 3-hour infusion to compensate for its rapid metabolic clearance and because steady-state plasma levels are not achieved before ~45 minutes.²³ The 3-hour infusion is also thought to reduce cerebellar toxicity associated with short-term administration.^{24,25} The number of HD-Ara-C infusions was restricted to 8 to avoid the sustained nonhematologic side effects reported for repetitive doses beyond 8 to 10.^{24,25} To take advantage of the previously mentioned conditioning effect of Ara-C, mitoxantrone was started on day 3 at 12 mg/m²/day and was repeated on days 4 and 5. In subsequent patients the total dose was escalated to 10 mg/m²/day for 5 days, which is in the range of the most effective single-agent regimens.¹²⁻¹⁶

Patients were eligible for the HAM regimen only if they were refractory to prior treatment.²⁶ Hence, they had either failed or two TAD-9 courses for induction treatment, had relapsed within the first 6 months on monthly maintenance including conventional dose Ara-C or after TAD-9 consolidation, were nonresponders to an additional TAD-9 course in later occurring first relapses, or had second or subsequent recurrences after successful TAD-9 therapy at the preceding relapse.

MATERIALS AND METHODS

Between January 1985 and March 1986, 40 patients entered the study from seven centers in Germany. Their ages ranged from 18 to 66 years (median 45 years). All patients had received one to two courses of the TAD-9 regimen for induction, consisting of thioguanine 200 mg/m²/day on days 3 to 9, Ara-C 100 mg/m²/day by continuous infusion on days 1 and 2 followed by short-term infusions of Ara-C 100 mg/m² every 12 hours on days 3 to 8, and daunorubicin 60 mg/m²/day on days 3 to 5 (TAD-9). For postinduction treatment, patients received TAD-9 consolidation and/or monthly maintenance therapy with subcutaneous Ara-C alternately combined with daunorubicin, thioguanine, or cyclophosphamide.^{21,22}

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Besides Ara-C at conventional dosage, all patients were therefore exposed to daunorubicin at cumulative doses between 180 and 990 mg/m² (median 450 mg/m²). All patients were refractory to conventional therapy as defined earlier (Table 1).

In three cases, AML was preceded by myelodysplastic syndrome. Two additional patients had secondary leukemias after radiation and chemotherapy for breast cancer and malignant seminoma, respectively. Four patients with early relapses had central nervous system (CNS) involvement.

Prior to therapy all patients gave informed consent after having been advised about the purpose and investigational nature of the study as well as of potential risks.

Therapy consisted of HD-Ara-C 3 g/m² every 12 hours by a 3-hour infusion on days 1 through 4. Mitoxantrone was started at 12 mg/m²/day as a 30-minute infusion on days 3, 4, and 5 and was escalated in subsequent cases to four and five doses of 10 mg/m²/day on days 2 through 5 and 2 through 6, respectively (Fig 1). For the prophylaxis of HD-Ara-C-induced photophobia and conjunctivitis, all patients received glucocorticoid eye drops every 6 hours starting before the first dose and continuing for 24 hours after the last dose of HD-Ara-C.

Toxicity was quantitated according to the World Health Organization (WHO) grading system.²⁷ For the most frequently encountered side effects, toxicity grades 3 and 4 are thereby defined as follows: nausea and vomiting—vomiting requiring therapy (grade 3); intractable vomiting (grade 4); diarrhea—repetitive diarrhea for >2 days requiring therapy (grade 3); severe hemorrhagic diarrhea with dehydration (grade 4); mucositis—ulcers requiring liquid diet only (grade 3); alimentation impossible, parenteral nutrition required (grade 4); and hepatic—increase in liver enzymes or bilirubin to 5 to 10 times the normal values (grade 3) or to >10 times the normal values (grade 4). In addition, cardiac function was monitored in all patients by echocardiographic measurement of left ventricular shortening fraction prior to therapy and at 4- to 6-week intervals thereafter.

Evaluation of antileukemic efficacy was based on CALGB criteria.^{28,29} Complete remission was defined by the disappearance of leukemic blasts from the bone marrow and blood as well as from possible extramedullary sites, including the cerebral fluid, and the normalization of peripheral blood counts to thrombocytes >100,000/ μ L and granulocytes >1,500/ μ L. The duration of critical cytopenia was evaluated by the time for granulocyte recovery to

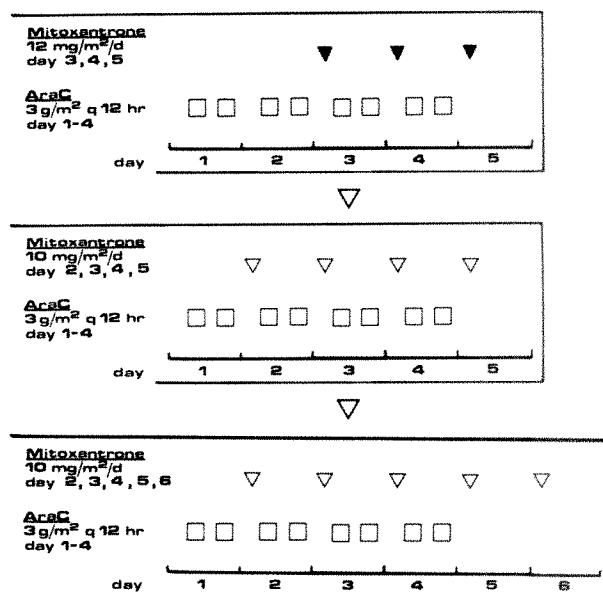


Fig 1. Schema of the high-dose cytosine arabinoside (HD-Ara-C)/mitoxantrone (HAM) protocol indicating the uniform dosage of HD-Ara-C 3 g/m² every 12 hours on days 1 through 4 and a dose escalation for mitoxantrone from 12 mg/m²/day on days 3 through 5 to four and five doses of 10 mg/m²/day on days 2 through 5 and 2 through 6.

>500/ μ L and thrombocytes to >20,000/ μ L from the onset of treatment.

RESULTS

Thirty-seven of the 40 patients were treated with one course of HAM, and three patients received a second cycle of the same regimen, accounting for 43 treatment courses. Eight cycles were carried out at the starting dose of mitoxantrone of 12 mg/m²/day $\times$ 3. In 16 and 19 courses, respectively, mitoxantrone was escalated to 4 and 5 doses of 10 mg/m²/day.

Nonhematologic toxicity. A summary of the nonhematologic side effects is given in Table 2. Nausea, vomiting, diarrhea, and mucositis were the most frequent side effects, occurring in 39 (91%), 35 (81%), and 30 (69%) of the 43 treatment courses. Severe side effects with toxicity grades of 3 and 4 according to WHO criteria were seen only in 9 (21%), 9 (21%), and 7 (16%) patients. Apart from more pronounced mucositis at the highest dose of mitoxantrone,

Table 1. Patient Characteristics and Entry Criteria

Characteristics	n	40
Age	18–66 yr	(median 45 yr)
Sex		
F		21
M		19
FAB subtypes		
M1		3
M2		14
M3		—
M4		13
M5		9
M6		1
Previous dose of daunorubicin	180–990 mg/m ²	
Entry Criteria	n	
Primary nonresponse against two TAD-9 induction cycles	10	
Early relapse within first 6 months	13	
TAD-9-resistant late relapse	8	
Second and higher relapse	9	

Table 2. Nonhematologic Toxicity

Toxicity	n (%)	WHO Grade I/II (%)	WHO Grade III/IV (%)
Nausea/vomiting	39 (91)	30 (70)	9 (21)
Diarrhea	35 (81)	26 (60)	9 (21)
Mucositis	30 (70)	23 (53)	7 (16)
Hepatic	11 (26)	9 (21)	2 (5)
Cardiac	8 (19)	5 (12)	3 (7)
CNS	6 (14)	2 (5)	4 (9)
Skin	5 (12)	5 (12)	—
Eye	4 (9)	4 (9)	—
Renal	3 (7)	3 (7)	—

Numbers and corresponding percentages in parentheses refer to all 43 treatment courses (100%).

Table 3. Entry Criteria and Treatment Response to HAM

Response	CR	PR	ED	NR	
Primary nonresponse	1	—	6*	3	10
Early relapse (<6 months CR)	9	1	2	1	13
TAD-9-resistant late relapse	3	—	5†	—	8
Second and higher relapses	8	—	—	1	9
	21	1	13	5	40

HAM, HD-Ara-C and mitoxantrone; CR, complete remission; PR, partial remission; ED, early death; NR, non-response.

*Includes one death due to pericardiac effusion.

†Includes one death due to acute cardiomyopathy.

toxicity was not different among the three applied dose escalations.

Two fatal cardiac events occurred at the lowest dose of mitoxantrone. One 31-year-old woman patient who had received 360 mg/m² of daunorubicin during prior therapy died of sudden cardiac failure 12 hours after the first dose of mitoxantrone. Autopsy revealed an acute cardiomyopathy. The other patient succumbed to a massive pericardiac effusion because of leukemic infiltration. Atrial flutter was observed in a third patient with preexisting cardiac arrhythmias during the initial HD-Ara-C infusion but was easily controlled by antiarrhythmic medication and did not cause cessation of treatment. Minor arrhythmias not requiring therapy occurred in two patients; in three additional patients, a reduction of left ventricular shortening fraction was revealed 3 to 8 weeks after HAM without any clinical signs of cardiac insufficiency.

In addition to two patients with transient dysdiadochokinesia, four patients aged 54, 59, 60, and 64 years had more severe CNS symptoms consisting of dysarthria, ataxia, balance disturbances, and somnolence. The clinical symptoms were completely reversible within 2 to 3 weeks in two patients; the other two died of septicemia during bone marrow aplasia.

Hematologic effects and antileukemic efficacy. Twenty-one of the 40 patients (53%) achieved complete remission (CR), 19 of them after one HAM course and 2 of them after two HAM courses. One additional patient obtained a partial remission (PR) with a normalization of peripheral blood counts but a residual leukemic cell population in the bone marrow of 15% blasts.

In five patients (13%), the leukemic blasts persisted or recovered after bone marrow aplasia; they were therefore considered nonresponders.

Three of the four patients with initial CNS involvement had no evidence of residual leukemic blasts in the cerebral fluid 1 week after completion of HAM without any intrathecal therapy and went into CR; the fourth patient died in aplasia due to an overwhelming infection.

Thirty-three of the 40 patients developed fever during bone marrow aplasia, and 11 (28%) died of infections between days 7 and 36 (median day 14) from the onset of therapy. Posttreatment bone marrow smears were available in nine cases and revealed residual leukemic blasts in only one patient. The two additional deaths caused by acute cardiomyopathy and pericardiac effusion occurred on days 2 and 7, respectively.

Analysis of response in relation to the previously defined entry criteria (Table 3) revealed similarly high CR rates in patients with early relapses (9 of 13) in TAD-9-resistant late relapses (3 of 8) and in patients with second and subsequent leukemic recurrences (8 of 9). Of the ten primary nonresponders only one patient achieved a CR. This group includes, however, two cases with antecedent myelodysplastic syndromes, two with additional ineffective salvage treatment by COAP or Aclacinomycin A and VP 16 and the patient who died of a pericardiac effusion (Table 4).

In patients achieving a CR ($n = 21$) or PR ($n = 1$), the median recovery time of granulocyte counts to $>500/\mu\text{L}$ and thrombocyte counts to $>20,000/\mu\text{L}$ was 27 days (range 21 to 150 days) from the start of treatment. The median time to complete remission was 36 days (range 24 to 190 days). No cases of prolonged aplasia occurred except for one patient at third relapse who had been exposed to previous multiple intensive regimens and who fulfilled CR criteria after 190 days.

Of the 21 CR patients, two underwent allogeneic bone marrow transplantations after 1⁺ and 2⁺ months of CR and both are presently in continuous CR at 16⁺ and 20⁺ months. Four patients are in ongoing remission at 1⁺ to 7⁺ months, whereas 15 relapsed after 1 to 8 months. The median remission duration is 4.5 months. Of the 15 relapsed patients, 8 were successfully reinduced with a modified HAM regimen, and 7 were refractory or died. The overall survival

Table 4. Characteristics of Primary Nonresponders Against Two TAD-9 Courses

Age (yr)	Antecedent Hematological Disorder	Additional Pretreatment	HD-AraC/Mitoxantrone Dose	Response	Cause of Death
46	—	—	I	ED day 36	Infection
47	—	5 × COAP	I	NR	—
59	—	—	II	ED day 5	Pericardiac effusion
51	—	—	2 × II	ED day 7	Infection
				Second course	
66	—	Acla/VP 16	II	NR	—
55	—	—	III	NR	—
50	—	—	III	ED day 14	Infection
59	Sideroachrestic anemia	—	III	ED day 35	Infection
60	Sideroachrestic anemia	—	III	ED day 19	Infection
67	—	—	III	CR	—

times are 3 months for all patients and 9 months for the 21 CR cases.

DISCUSSION

In refractory and relapsed acute leukemias, clinical phase I/II studies are performed not only as salvage therapy for patients with advanced disease but also as a search for new agents or drug combinations with antileukemic activity that will broaden the spectrum of leukemia treatment and may subsequently be incorporated into first-line therapy. The clinical evaluation of these trials and the interstudy comparison of treatment results are substantially hampered, however, by the great heterogeneity of patients entered on phase I/II protocols. In addition to various forms of first-line therapy, differences in the duration of the preceding remission and in the definition of patient eligibility may cause divergent results and must be considered.^{30,31}

The present phase I/II study combining HD-Ara-C and mitoxantrone (HAM) was based on the first-line multicenter trial of the German AML Cooperative Group²² and was thus applied under standardized conditions for the initial treatment by TAD-9, producing a 62% CR rate in 844 adults of all ages. Furthermore, rigid criteria for the definition of refractoriness against first-line therapy were applied according to the analysis of relapse pattern and response to reinduction attempts with the TAD-9 regimen initially used. This evaluation indicated a substantially inferior remission rate in patients relapsing within the first 6 months (19% CR) as compared with later relapses (45% CR). TAD-9 was also ineffective in cases with second and subsequent leukemic recurrences producing 21% CR. Hence, patients were eligible for HAM only if they had either failed on induction therapy, relapsed within 6 months during intensive monthly maintenance treatment or after a full-dose induction-type consolidation, were nonresponders to a reinduction attempt with TAD-9 at later recurrences, or were at second or subsequent relapses. Under these prerequisites, the response rate of 53% CR demonstrates a substantial antileukemic activity of HAM in refractory AML.

Willemze and colleagues¹ treated 14 similarly selected patients by a six-day course of HD-Ara-C alone and obtained 6 CR and 1 additional hematologic CR with persistent extramedullary leukemia; however, they reported considerable pulmonary toxicity in three patients. This side effect was not observed by Herzig and co-workers at the same dose level,² with which 12 of 19 evaluable patients with AML achieved a CR. Five patients dying during the first 3 weeks were considered invaluable, and the responses were not classified according to prior therapy or relapse status. The overall study comprised 36 patients who had received different types of primary treatment and of whom only 14 were considered refractory against conventional-dose Ara-C. In a recent report by the same group,³ 3 of 15 patients with AML resistant to conventional-dose Ara-C responded to 12 doses of HD-Ara-C whereas addition of an anthracycline produced 15 CR in 27 similarly selected AML patients. Remission rates for combinations of HD-Ara-C with *m*-AMSA range from 33% to 70%.^{4,6,32,33} These trials are hardly comparable, however, because of different drug schedules and doses. In addition, information about the prior therapy and eligibility

criteria including the resistance to conventional-dose Ara-C are not available. The latter data are given for two recent trials with sequential HD-Ara-C and asparaginase in children and adults^{3,34} achieving CR in 42% to 68% of cases. The earlier study³ also included patients without prior Ara-C treatment; in both trials, patients with late relapses were eligible if they were still receiving maintenance therapy.

These data emphasize the limits of interstudy comparison but strongly suggest that HAM is at least equivalent or possibly even superior to other HD-Ara-C regimens as well as to the combination of mitoxantrone and VP-16³⁵ in truly refractory AML. Preliminary results of a recent study using HD-Ara-C and mitoxantrone at a different dosage and timing confirm the high overall remission rate, although 4 of 10 patients needed more than one treatment cycle to obtain a CR.³⁶

Analysis of treatment response according to the previously defined entry criteria revealed high remission rates in patients with early relapses and TAD-9-resistant late relapses as well as in second and subsequent recurrences, but only one CR in ten patients with primary resistance against two TAD-9 induction cycles. Although additional factors such as antecedent myelodysplastic syndromes and further salvage therapy must be taken into account in four patients, these results seem to imply that primary refractoriness against two TAD-9 cycles might not be overcome by HAM. Due to the relatively small number of truly evaluable primarily resistant patients, this conclusion must be considered preliminary and deserves confirmation by additional cases.

Despite intensive prior therapy and exposure to a median cumulative dose of 450 mg/m² of daunorubicin (range 180 to 990 mg/m²), the toxicity of HAM was acceptable at all dose levels. The only cardiac death that was obviously related to therapy consisted of an acute cardiomyopathy in a 31-year-old patient without a prior history of cardiac disorder who had previously received 360 mg/m² of daunorubicin. Two cases of minor arrhythmias and one incidence of atrial flutter were observed during the first infusion of HD-Ara-C, as has been described by others,¹ and were easily controlled by antiarrhythmic medication. A second cardiac death was due to a massive pericardiac effusion because of leukemic infiltration and was not related to treatment toxicity. Three other patients developed a reduction of left ventricular shortening fraction 3 to 8 weeks after HAM without any clinical signs of cardiac insufficiency. The incidence and severity of CNS toxicity are comparable with that of other HD-Ara-C regimens,^{4,5,34} as are the hematologic effects of HAM. Blood counts recovered to granulocytes $\geq 500/\mu\text{L}$ and thrombocytes $>20,000/\mu\text{L}$ at a median of 27 days after the onset of therapy, and the median time to complete remission was 36 days. Corresponding recovery times are reported for combinations of HD-Ara-C with anthracyclines, *m*-AMSA, and asparaginase.^{3,5,6,34}

The overall early death rate of 33%, which certainly exceeds the results of some other HD-Ara-C trials, must be judged in consideration of the rigid eligibility criteria for the present study. Patients with primarily refractory AML or nonresponse to TAD-9 at late occurring relapses are in continuous hypoplasia or aplasia for 4 to 8 weeks before

entering the HAM protocol. Therefore, they carry a high risk of acquiring severe infections, the major cause of death in 11 of the 13 cases. Ten of the 13 early deaths were observed in patients of the two mentioned subgroups. In this context, the low rate of 12% HAM-resistant leukemias must be emphasized as well; the rate compares favorably with those of other

HD-Ara-C or mitoxantrone combinations.^{6,34,35} Hence, despite selection of heavily pretreated patients with advanced disease and poor prognosis, HAM produced an encouraging high overall response rate, which strongly suggests incorporating the two-drug combination at earlier stages of AML treatment.

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Characterization of Glucocorticoid Receptors and Glucocorticoid Receptor mRNA in Human Leukemia Cells: Stabilization of the Receptor by Diisopropylfluorophosphate

By Clark W. Distelhorst and Roger Miesfeld

We have shown that cytosol samples from human leukemia cells frequently contain glucocorticoid receptor fragments that have a mol wt (M_r) of $\sim 52,000$. In the present study we demonstrate that the $M_r \sim 52,000$ -receptor fragments are derived from intact glucocorticoid receptors ($M_r \sim 97,000$) by the action of a serine protease. $M_r \sim 52,000$ -receptor fragments were present in cytosol from 24 of 52 leukemia cell samples. Only normal size glucocorticoid receptors were present in cytosol samples if diisopropylfluorophosphate (DFP), a potent inhibitor of serine proteases, was added to the hypotonic buffer used for cytosol preparation. Receptor proteolysis was not inhibited by hydrolyzed DFP, benzamidine, phenylmethylsulfonylfluoride, aprotinin, iodoacetamide, or mercuric chloride. The leukemia cell protease digests the receptor at a different site than chymotrypsin, which digests the intact receptor to produce a

$M_r \sim 40,000$ receptor fragment. Receptor messenger RNA (mRNA) in S49 mouse lymphoma cells and in human leukemia cells was analyzed by Northern hybridization with a cDNA for the normal glucocorticoid receptor. Mutant S49 mouse lymphoma cells that have abnormally small glucocorticoid receptors ($M_r \sim 48,000$) make a 5.0-kilobase receptor transcript in addition to the normal size 6.5-kilobase receptor transcript. A normal size receptor transcript of 6.5 kilobases was present in all of the human leukemia cells whether or not $M_r \sim 52,000$ -receptor fragments were present. Therefore, abnormalities of glucocorticoid receptor mRNA, which may give rise to the synthesis of foreshortened receptors in certain mutant mouse lymphoma cells, are apparently absent from human leukemia cells.

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THE EFFECTS of glucocorticosteroids on cells are mediated through receptors that bind the steroid hormone molecule to form a steroid-receptor complex.¹ The steroid-receptor complex then interacts with specific regions in the genome to regulate gene transcription.² Glucocorticoids kill leukemia and lymphoma cells and, therefore, are an important therapeutic modality for leukemias and lymphomas. Although the exact mechanism of cell killing by glucocorticoids is unknown, studies of glucocorticoid-sensitive and glucocorticoid-resistant mouse lymphoma cell lines have demonstrated that the cell killing process is mediated through glucocorticoid receptors and that a variety of receptor defects can result in glucocorticoid resistance.³

Because of the importance of glucocorticoids in the therapy of human leukemias and lymphomas, there is considerable interest in the structural and functional properties of glucocorticoid receptors in human leukemia and lymphoma cells. Recent studies have found that glucocorticoid receptors

in many human leukemia cells are abnormal in terms of their physical properties and their ability to bind to DNA cellulose or diethylaminoethyl (DEAE) cellulose.^{4,5} Glucocorticoid receptors in cytosol from human leukemia cells are subject to digestion by endogenous proteases.⁵⁻⁸ Thus it has been suggested that previously described abnormalities of glucocorticoid receptor function could be due to the presence of receptor fragments in leukemia cell cytosol and that glucocorticoid receptors in most, if not all, human leukemia cells are normal in size prior to digestion by endogenous proteases.^{5,7}

The mechanisms of glucocorticoid receptor degradation in cytosol from both normal cells and leukemia cells is currently being studied by several groups of investigators. Sherman et al⁹ demonstrated proteolytic degradation of the intact glucocorticoid receptor in rat liver and kidney cytosol to the meroreceptor, which is the smallest receptor fragment ($M_r \sim 20,000$ to $25,000$) that retains the steroid binding site. The extent of receptor degradation appeared to correlate with the activity of "lysine-specific" endopeptidases.⁹ Both Sherman et al⁷ and Holbrook et al⁵ demonstrated the presence of meroreceptors in cytosol from human leukemia cells. The specific protease(s) responsible for receptor degradation in human leukemia cell cytosol have not been identified. However, Sherman et al⁷ found high activities of peptidases of various specificities in human leukemia cell cytosols and showed that a lysine-specific endopeptidase isolated from rat kidney cytosol is capable of converting the glucocorticoid receptor in chronic lymphocytic leukemia cells to a meroreceptor.¹⁰ Holbrook et al⁸ found that glucocorticoid receptors from acute nonlymphocytic leukemia cells could be stabilized by a factor from chronic lymphocytic leukemia cells. Therefore receptor degradation to the meroreceptor form is influenced not only by the presence or absence of receptor degrading proteases but also by the level of factors in leukemia cell cytosol that inhibit receptor proteolysis.⁸

We recently found that cytosol from human leukemia cells frequently contains a $M_r \sim 52,000$ -receptor fragment.¹¹ The

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$M_r \sim 52,000$ -receptor fragment was not unique to a particular type of leukemia and was present in samples from previously untreated and previously treated patients.¹¹ This receptor fragment is considerably larger than the meroreceptor fragment described previously by others (see above) and corresponds closely in size to the nuclear transfer increased (nt^1) mutant-glucocorticoid receptor in mouse lymphoma cells.¹²⁻¹⁸ These mutants contain a receptor mRNA that is reduced in size and therefore results in synthesis of a foreshortened receptor molecule.¹⁹

The present study was undertaken to determine the mechanism responsible for the $M_r \sim 52,000$ -receptor fragment in human leukemia cell cytosol. Our studies demonstrate for the first time that human leukemia cells contain a serine protease that digests the intact glucocorticoid receptor to a $M_r \sim 52,000$ -fragment. We find that glucocorticoid receptors in over 50 different leukemia cell samples are normal in size if diisopropylfluorophosphate, a potent inhibitor of serine proteases, is used to stabilize the receptor. Consistent with this observation, we find that the receptor mRNA in human leukemia cells has a normal size.

METHODS

Materials. All chemicals, including proteases, protease inhibitors, and unlabeled dexamethasone, were purchased from Sigma Chemical Co., St. Louis. Acrylamide, N,N'-methylenebisacrylamide, and Coomassie blue R-250 were from Bio-Rad Laboratories, Richmond, CA. The low mol wt standards kit for gel electrophoresis was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. [6,7-³H]Dexamethasone-21-mesylate was from New England Nuclear, Boston.

Buffers. Phosphate buffered saline (PBS) was prepared as described previously.¹¹ The hypotonic buffer used in cytosol preparation consisted of 25 mmol/L Tris pH 8.2, 1 mmol/L EDTA, 10% glycerol, and 20 mmol/L Na₃MoO₄.

Cell Lines. The Victor human leukemia cell line (a B lymphoblast cell line) was given by Dr Robert Allen (American Red Cross, St. Louis). Mouse lymphoma cell lines and methods of cell culture were described previously.²⁰

Leukemia cell isolation. Human leukemia cells were isolated from either peripheral blood or bone marrow by Ficoll-Hypaque separation as described previously.¹¹ Only freshly isolated cells were used for experiments.

Affinity labeling. The method of labeling receptors in intact cells with [³H]dexamethasone mesylate was described in detail previously.¹¹ Briefly, cells were washed with PBS and resuspended in PBS in Eppendorf microcentrifuge tubes so that each tube contained 10 to 20 million cells. The cell suspensions were incubated for four hours at 4°C with 200 nmol/L [³H]dexamethasone mesylate. Duplicate cell suspensions contained either the radiolabeled steroid alone (labeled — in figures) or the radiolabeled steroid plus 100-fold excess unlabeled dexamethasone (labeled + in figures). At the end of the incubation period, the cells were pelleted and the cell pellets were frozen at -80°C for 15 minutes and then thawed in hypotonic buffer at 4°C for 15 minutes to prepare cytosol. The cytosol was then boiled for two minutes in the sample buffer for gel electrophoresis and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as detailed previously.¹¹ Gels were analyzed by autoradiography.¹¹ Molecular weight standards were phosphorylase b (97,400), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor

(20,100), and lactalbumin (14,400). References for the mol wt values were given previously.¹¹

Protease inhibitors. To inhibit proteolysis of the affinity-labeled receptor during cytosol preparation, protease inhibitors were added to the hypotonic buffer immediately prior to use. Diisopropylfluorophosphate (DFP) was added to the hypotonic buffer to give a final concentration of 5 mmol/L. To exclude the possibility that the inhibitory effect of DFP was due to an impurity present in certain commercial preparations of DFP, samples of DFP were treated with cupric ion and imidazole to catalyze the hydrolysis of DFP.^{21,22} In other experiments a stock solution of phenylmethylsulfonylfluoride (PMSF) was prepared in absolute ethanol and added to the hypotonic buffer to give a final concentration of 10 mmol/L. Benzamidine was added to the hypotonic buffer to give a final concentration of 5 mmol/L. Aprotinin (2 mg/mL; 14.5 trypsin inhibitory U/mg) was added to the hypotonic buffer at a final dilution of 1:1000. A 100 mmol/L stock solution of iodoacetamide was added to the hypotonic buffer to give a final concentration of 1 mmol/L. A stock solution of 0.01 mol/L HgCl₂ was added to the hypotonic buffer to give a final concentration of 0.01 mM. The hypotonic buffer was prepared without EDTA when HgCl₂ was used.

Limited protease digestion. A concentrated solution of chymotrypsin in deionized water was prepared fresh for each experiment. An aliquot of the chymotrypsin solution was added to affinity-labeled cytosol and incubated at 25°C for 15 minutes. The cytosol was transferred to the gel electrophoresis sample buffer and boiled to stop the reaction.

RNA isolation and analysis. Total cellular RNA was isolated from leukemia cells by sedimentation through cesium chloride.²³ The RNA was analyzed by Northern blot hybridization using a cDNA for the rat liver glucocorticoid receptor as described previously.¹⁹

RESULTS

Affinity labeling human leukemia cell receptors. Human leukemia cells were isolated from peripheral blood or bone marrow and then incubated with [³H]dexamethasone mesylate to affinity label the glucocorticoid receptors. Cytosol was then prepared and subjected to SDS-PAGE to determine the mol wt of the affinity-labeled receptors. Multiple proteins in cells bind [³H]dexamethasone mesylate and therefore appear as bands on autoradiograms of gels. To distinguish the specific binding proteins, or receptors, from the nonspecific binding proteins, duplicate cell suspensions were incubated with [³H]dexamethasone mesylate in the presence or absence of 100-fold excess unlabeled dexamethasone. The unlabeled dexamethasone competes with the [³H]dexamethasone mesylate for binding to receptors but does not compete for binding to nonsaturable binding sites on other proteins. Therefore, on autoradiograms, band(s) that correspond to affinity-labeled receptor(s) can be distinguished from nonreceptor bands because the receptor bands are seen only in the absence of competing steroid (lane —) and are not seen in the presence of competing unlabeled steroid (lane +). Small differences in the amount of cytosol applied to the — lane and + lane on gels could give rise to apparent differences in the intensity of corresponding bands on the two lanes. Therefore, only those bands that differ markedly in intensity between — and + lanes are considered to be receptor bands. For clarity, receptor bands on autoradiograms are designated by an arrowhead.

We determined the mol wt of glucocorticoid receptors in

cytosol from 52 different human leukemia samples. Cytosol from all of the samples contained intact receptors that migrated opposite the phosphorylase b marker and therefore have been assigned a M_r of 97,000 (Fig 1). A major receptor fragment that consistently migrated to just above the ovalbumin standard ($M_r \sim 45,000$) was detected in cytosol from 24 of the 52 leukemia samples, as illustrated in Fig 1. We previously assigned this receptor fragment a M_r of 55,000.¹¹ We have now reanalyzed the previously reported results along with additional experiments reported here and find the M_r of this receptor fragment to be $52,000 \pm 500$ (mean $\pm$ SE, $N = 24$). Although the M_r 52,000-receptor fragment was the major receptor fragment detected in our studies, we cannot exclude the possibility that other minor receptor fragments may be present in the cytosol of leukemia cells. For example, a $M_r \sim 30,000$ -receptor fragment appeared to be present in the cytosol of three leukemia samples, one of which is shown in Fig 2.

$M_r \sim 52,000$ -receptor fragments were detected with approximately equal frequency in the different types of leukemia studied (Table 1). These receptor fragments were detected in the cytosol of leukemia cells from previously untreated patients (8/23, 35%) and previously treated patients (16/29, 55%).

Effect of protease inhibitors. To determine if the $M_r \sim 52,000$ -receptor is present within intact cells or if it is produced by proteolytic digestion of $M_r \sim 97,000$ -receptor during cytosol preparation, 5 mmol/L diisopropylfluoro-

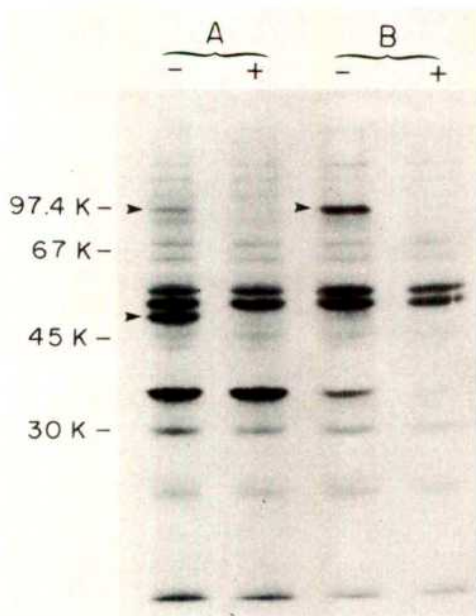


Fig 1. Effect of DFP on glucocorticoid receptors. Human leukemia cells (acute lymphocytic leukemia) were incubated with [^{3} H]dexamethasone mesylate in the absence (-) or presence (+) of excess unlabeled dexamethasone to affinity label the glucocorticoid receptors. Cytosol was prepared using hypotonic buffer that either did not contain DFP (A) or did contain DFP (B). The cytosol was subjected to SDS-PAGE, and the gels were analyzed by autoradiography, as shown here. Arrowheads point to bands that represent affinity-labeled receptors.

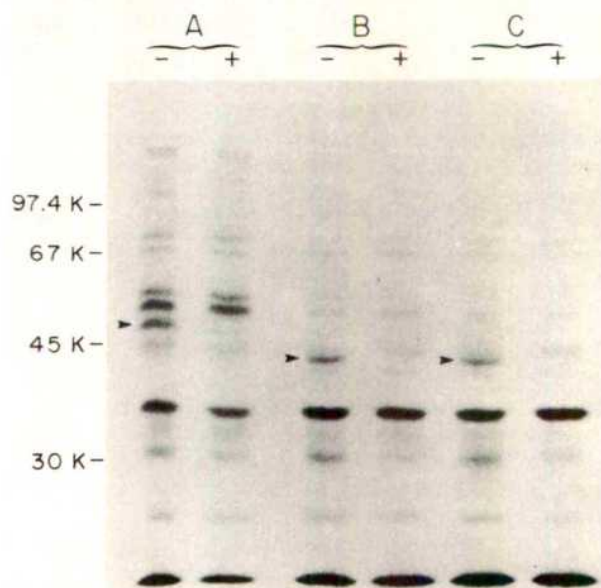


Fig 2. Effect of chymotrypsin on glucocorticoid receptors. Same as Fig 3 except that leukemia cells from a different patient (chronic lymphocytic leukemia) were studied. (A) No chymotrypsin. (B) 5 μ g/mL chymotrypsin. (C) 10 μ g/mL chymotrypsin. Arrowheads point to bands that represent affinity-labeled receptor fragments. An additional receptor fragment appears to be located above the 30k marker protein.

phosphate (DFP) was included in the hypotonic buffer used for cytosol preparation. In cytosol samples that contained only $M_r \sim 97,000$ -receptors, the intensity of the receptor band on autoradiograms was not affected by inclusion of DFP in the hypotonic buffer (not shown). In samples that contained both $M_r \sim 97,000$ - and $M_r \sim 52,000$ -receptors, the inclusion of DFP in the hypotonic buffer eliminated the $M_r \sim 52,000$ -receptor band and increased proportionately the intensity of the $M_r \sim 97,000$ -receptor band (Fig 1). This finding indicates that the $M_r \sim 52,000$ -receptor band represents a receptor fragment produced from the $M_r \sim 97,000$ -receptor by proteolytic digestion during cytosol preparation. The ability of DFP to eliminate the $M_r \sim 52,000$ -receptor band was confirmed in ten separate experiments using leukemia cells from a different patient in each experiment. In no experiment did DFP fail to prevent formation of the $M_r \sim 52,000$ -receptor.

To determine the type of protease responsible for generat-

Table 1. Glucocorticoid Receptors in Human Leukemia Cells

Type of Leukemia	Receptor M_r		
	97,000	97,000 + 52,000	Total
Acute lymphoblastic	9	8	17
Acute nonlymphoblastic	9	8	17
Chronic lymphocytic	8	7	15
Chronic myelogenous, blast crisis	2	1	3

Values represent the number of leukemia cell samples that contained either $M_r \sim 97,000$ receptors or both $M_r \sim 97,000$ and $M_r \sim 52,000$ receptors.

ing $M_r \sim 52,000$ -receptor fragments in leukemia cytosol, a variety of protease inhibitors with different protease specificities were included in the hypotonic buffer used for cytosol preparation. Inhibitors that have major specificity for serine proteases (PMSF, aprotinin, and benzamide) and inhibitors that have major specificity for cysteine proteases (HgCl_2 and iodoacetamide) did not inhibit receptor digestion (not shown). Certain preparations of DFP are contaminated with a factor that inhibits cysteine proteases.^{21,22} The serine protease inhibitory activity of DFP is eliminated by hydrolysis, but the cysteine protease inhibitory activity is stable upon hydrolysis.^{21,22} DFP that was hydrolyzed to eliminate its serine protease inhibitory activity did not block the generation of $M_r \sim 52,000$ -receptors (not shown). In addition, in studies that are not shown, DFP did not inhibit receptor digestion by the cysteine protease papain. Therefore, the protease responsible for digestion of the intact leukemia cell receptor to a $M_r \sim 52,000$ -fragment appears to belong to the serine class of proteases.

Limited protease digestion. Limited digestion of normal size glucocorticoid receptors with chymotrypsin is known to separate the immunoactive domain from the steroid-binding and DNA-binding domains of the receptor molecule.^{24,25} To determine if the endogenous protease in human leukemia cells digests the glucocorticoid receptor at the same site as chymotrypsin, [^3H]dexamethasone mesylate-labeled cytosol from human leukemia cells was incubated with a range of concentrations of chymotrypsin prior to gel electrophoresis. In cytosol that contained only $M_r \sim 97,000$ -receptors, chymotryptic digestion produced a $M_r \sim 40,000$ -receptor fragment (Fig 3). In cytosol that contained both $M_r \sim 97,000$ -

and $M_r \sim 52,000$ -receptors, chymotryptic digestion eliminated both the $M_r \sim 97,000$ - and the $M_r \sim 52,000$ -receptors and produced a $M_r \sim 40,000$ -receptor fragment (Fig 2). These results indicate that the endogenous leukemia cell protease digests the glucocorticoid receptor at a different site than does chymotrypsin and that the $M_r \sim 52,000$ receptor fragment in leukemia cytosol contains the chymotrypsin sensitive site that is present in the intact $M_r \sim 97,000$ receptor.

Mixing studies. To further substantiate that proteolysis was responsible for producing the $M_r \sim 52,000$ receptor fragment, cells of the Victor human leukemia cell line were incubated with [^3H]dexamethasone mesylate and then mixed with unlabeled fresh human leukemia cells immediately prior to cytosol preparation. The human leukemia cells were preincubated with $20 \mu\text{mol/L}$ unlabeled dexamethasone to prevent their receptors from binding the [^3H]dexamethasone mesylate. A typical experiment is shown in Fig 4. This experiment employed the same human leukemia cell sample that was employed in the experiment shown in Fig 2. In the absence of added human leukemia cells, cytosol from the Victor cells contained only $M_r \sim 97,000$ -receptors. The addition of the unlabeled human leukemia cells to the affinity-labeled Victor cells prior to cytosol preparation generated $M_r \sim 52,000$ -receptor fragments from the intact affinity-labeled receptors.

The leukemia cell samples employed in this study contained variable numbers of red blood cells and platelets. To

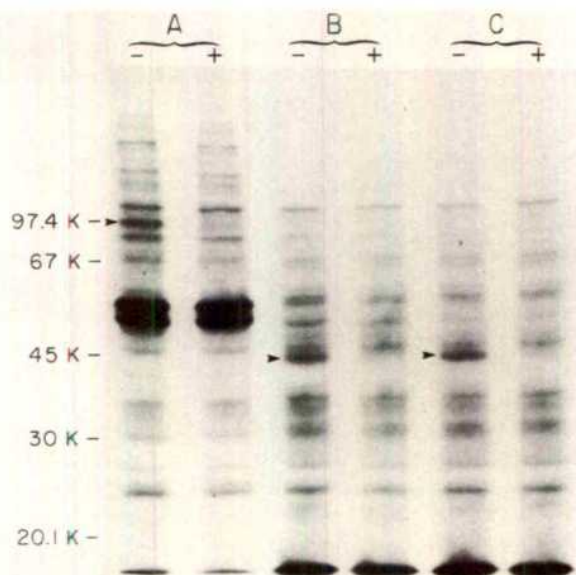


Fig 3. Effect of chymotrypsin on glucocorticoid receptors. Receptors in human leukemia cells (chronic lymphocytic leukemia) were affinity labeled as described in Fig 1. The cytosol was incubated at 25°C for 15 minutes with chymotrypsin prior to SDS-PAGE. (A) No chymotrypsin. (B) $5 \mu\text{g/mL}$ chymotrypsin. (C) $10 \mu\text{g/mL}$ chymotrypsin. Arrowheads point to bands that represent affinity-labeled receptors or receptor fragments.

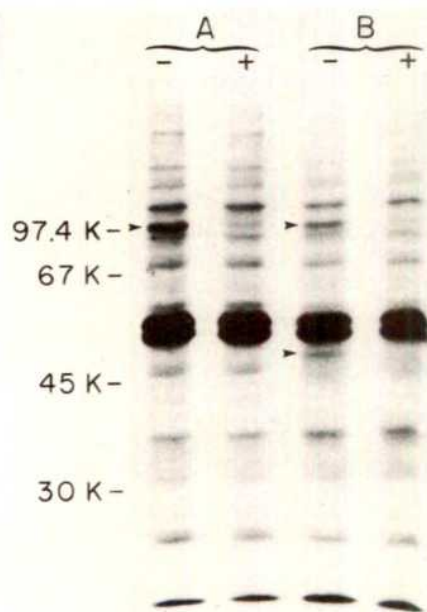


Fig 4. Mixing studies. Glucocorticoid receptors in Victor cells were affinity-labeled as described in Fig 1. Leukemia cells (chronic lymphocytic leukemia) from the same sample used in Fig 2 were incubated for four hours with $20 \mu\text{mol/L}$ unlabeled dexamethasone. The unlabeled cells were then mixed with the affinity-labeled Victor cells in a ratio of 3:1 immediately prior to cytosol preparation. Cytosol was then analyzed by SDS-PAGE as described in Fig 1. (A) Victor cells with no addition. (B) Victor cells plus unlabeled leukemia cells. Arrowheads point to bands that represent affinity-labeled receptors or receptor fragments.

determine if red blood cells or platelets, rather than leukemia cells, could be the source of the protease that digests receptors to the $M_r \sim 52,000$ -fragment, experiments were performed in which human red blood cells or human platelets were added to affinity-labeled leukemia cells prior to cytosol preparation. The admixture of these cells did not produce receptor degradation (data not shown).

Receptor mRNA. Receptor mRNA from human cell samples was compared to receptor mRNA from mouse lymphoma cell lines. Glucocorticoid receptor mRNA in six different human leukemia cell samples was analyzed by Northern blot hybridization using a receptor cDNA for the rat liver glucocorticoid receptor, and results from four of these samples are shown in Fig 5. A normal 6.5-kilobase (kb) receptor mRNA was observed in human cells that contain both normal size receptors and the $M_r \sim 52,000$ -fragments. The patient samples containing $M_r \sim 52,000$ -receptor fragments (lanes 5, 6, 7) have receptor mRNA that looks identical to the mRNA from the patient sample containing only intact receptors (lane 2). The faintness of the 6.5 kb bands in lanes 5 and 7 may reflect lower levels of receptor

mRNA in these leukemia samples or degradation of the RNA during preparation. The wild type S49 mouse lymphoma cells, which have normal size ($M_r \sim 97,000$) glucocorticoid receptors, have a 6.5 kb receptor mRNA (lane 4). The nt^1 mutant mouse lymphoma cells (S49.143r), which have smaller than normal glucocorticoid receptors ($M_r \sim 48,000$), have a 5.0 kb receptor mRNA. We conclude that the receptor mRNA in human leukemia cells is the expected size regardless of the mol wt of receptors in cytosol preparations, unlike the highly characterized 5.0 kb receptor mRNA present in nt^1 mutant mouse lymphoma cells.¹⁹

DISCUSSION

We demonstrate that cytosol from human leukemia cells frequently contains steroid binding receptor fragments that have a M_r of $\sim 52,000$ in addition to intact receptors that have a M_r of $\sim 97,000$. Several samples also appeared to contain $M_r \sim 30,000$ -receptor fragments. The present study provides two types of evidence that prove that the $M_r \sim 52,000$ -receptor fragment arises from the intact normal size receptor by proteolytic digestion during cytosol preparation. First, formation of the $M_r \sim 52,000$ -receptor fragment was blocked by inclusion of the potent protease inhibitor DFP in the hypotonic buffer used for cytosol preparation. Second, admixture of fresh unlabeled human leukemia cells to an affinity-labeled human leukemia cell line prior to cytosol preparation resulted in digestion of the intact affinity-labeled receptors to $M_r \sim 52,000$ -receptor fragments. We previously reported similar mixing studies to be negative.¹¹ This led us to the incorrect conclusion that proteolysis was not responsible for the formation of $M_r \sim 52,000$ -receptor fragments in the cytosol of human leukemia cells. The previous mixing studies involved the mixture of cytosol samples that had been stored at -80°C rather than the mixture of fresh intact cells as in the present study. Therefore the failure of the earlier mixing studies to demonstrate receptor proteolysis suggests that the leukemia cell protease responsible for receptor digestion may not be stable upon storage at low temperatures.

Limited digestion of the normal glucocorticoid receptor by chymotrypsin is known to separate the immunoactive domain of the receptor from the steroid-binding and DNA-binding domains.^{24,25} The leukemia cell protease described here must digest the intact $M_r \sim 97,000$ glucocorticoid receptor at a different site than does chymotrypsin, since chymotryptic digestion of the intact receptor produced a $M_r \sim 40,000$ -receptor fragment (Fig 3). The $M_r \sim 52,000$ -receptor fragment in human leukemia cell cytosol could be digested to a $M_r \sim 40,000$ -receptor fragment by chymotrypsin, indicating that the $M_r \sim 52,000$ -receptor fragment retains the chymotrypsin sensitive site that is present in normal receptors (Fig 2).

The $M_r \sim 52,000$ -receptor fragment was detected in leukemia cell samples obtained from 24 of 52 different patients. The presence of the $M_r \sim 52,000$ -receptor fragment was consistent when leukemia cell samples were obtained again from the same patient at time intervals of up to 1 year. We previously demonstrated that receptor digestion does not occur in some leukemia cell cytosol samples even when

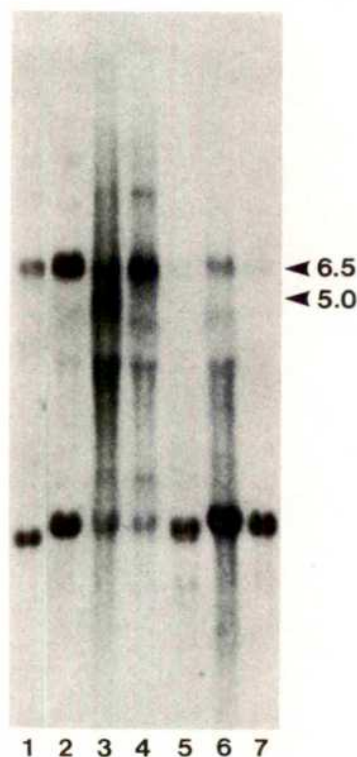


Fig 5. Northern blot analysis of leukemia cell receptor RNA. Total cellular RNA from five human samples (lanes 1, 2, 5-7) and two murine samples (lanes 3, 4) were blotted with a probe from the 5' region of the rat glucocorticoid receptor cDNA¹⁹ (Miesfeld et al, unpublished data). Lane 1 (HeLa cells), lane 2 (human leukemia cells), and lane 4 (mouse lymphoma cells S49.A2;wt) show the 6.5-kb receptor RNA present in cells containing only $M_r \sim 97,000$ receptors. Lanes 5, 6, 7 (human leukemia) and lane 3 (mouse lymphoma S49.143r; nt^1) contain RNA from cells that also possess a smaller receptor protein of $M_r \sim 48,000$ to $52,000$. The nt^1 -specific receptor RNA is shown as a 5.0-kb transcript (lane 3). The nature of the smear around 4 kb is unclear, but it appears to be due to partially degraded RNA.

proteolysis is encouraged by prolonged incubation or incubation at 25 °C to 37 °C.¹¹ The reason receptor digestion occurs in some but not all human leukemia cell samples is unknown. It is possible that the protease responsible for receptor degradation may not be present in all leukemia cells or that some leukemia cells may contain a naturally occurring protease inhibitor that protects the receptor from proteolysis. Alternatively, the protease activity may be similar in all leukemia cells but some leukemia cells may contain glucocorticoid receptors that are abnormally sensitive to digestion by the protease. We are attempting to distinguish between these possibilities at the present time. Receptor digestion does not occur in normal human peripheral blood mononuclear cell samples under the assay conditions employed here.¹¹ However, we have observed receptor digestion to a $M_r \sim 52,000$ -fragment when normal human peripheral blood mononuclear cell cytosol is incubated for two hours at 25 °C (data not shown). Receptor digestion under these conditions is inhibited by DFP. Therefore, the protease responsible for receptor digestion may not be unique to leukemia cells but appears to be present, although probably at a lesser activity, in normal peripheral blood mononuclear cells.

The $M_r \sim 52,000$ -receptor fragment described here is similar in size to the defective receptors present in cytosol from nt^1 mutant mouse lymphoma cell lines.^{13,16-18} This observation raised the possibility that the receptor abnormality in human leukemia cells and in the nt^1 mutant mouse lymphoma cells may involve a common mechanism.¹¹ In this regard there is some evidence that glucocorticoid receptors in intact nt^1 mutant cells are normal in size and undergo degradation during cytosol preparation.²⁶ However, the receptor abnormalities in human leukemia cells and in nt^1 mutant mouse lymphoma cells differ in several respects. First, a variety of different protease inhibitors, including DFP, do not inhibit the formation of defective receptors in cytosol from nt^1 mutant mouse lymphoma cells (Distelhorst et al, unpublished data). Second, the $M_r \sim 52,000$ -receptor in human leukemia cells contains a chymotrypsin-sensitive site, whereas the defective receptors in nt^1 mutant mouse lymphoma cells are relatively resistant to digestion by chymotrypsin¹⁷ (Distelhorst et al, unpublished observations). Third, the receptor mRNA in nt^1 mutant mouse lymphoma cells is decreased in size compared to the size of receptor mRNA in wild type cells.¹⁹ In the present study we show that the size of receptor mRNA in human leukemia cells appears to be normal whether or not the $M_r \sim 52,000$ -receptor fragment is observed. Therefore abnormalities of glucocorticoid receptor mRNA, which may give rise to the synthesis of foreshortened receptors in certain mutant mouse lymphoma cells, are apparently absent from human leukemia cells.

Stevens et al²⁷ showed that glucocorticoid receptors in chronic lymphocytic leukemia cells are often degraded to a form having a Stokes radius of 3.5 nm. According to the work of Sherman et al,⁹ a receptor fragment of this size would be expected to have a $M_r \sim 40,000$ to 60,000. Therefore it is possible that the $M_r \sim 52,000$ -receptor fragment described by us is the same as the lower mol wt form of the receptor described previously by Stevens et al.²⁷ Sherman et al⁷ and Holbrook et al^{5,8} found that cytosol from human leukemia

cells frequently contained meroreceptors in addition to normal size receptors. The meroreceptor, described by Sherman et al⁹ in rat liver and kidney cytosol, represents a degraded receptor that has a $M_r \sim 20,000$ to 25,000. Although similar size receptor fragments ($M_r \sim 30,000$) were detected in several leukemia samples studied here, the major receptor fragment observed in the present study was significantly larger ($M_r \sim 52,000$). There are two potential explanations for the difference in size between receptor fragments detected in the present study and earlier studies. First, we analyzed receptors under reducing and denaturing conditions by gel electrophoresis, whereas the size of receptors in earlier studies was measured under nondenaturing conditions by gel filtration. Mendel et al²⁸ demonstrated that gel electrophoresis under denaturing conditions is a more sensitive technique for detecting intermediate size receptor fragments ($M_r \sim 50,000$) than is gel filtration under nondenaturing conditions. Second, in the present study, cytosol samples were incubated for only a brief period of time (~ 30 minutes) prior to gel electrophoresis, whereas the assay methods employed in earlier studies required cytosol incubations for longer periods of time, thus permitting more extensive receptor degradation.

Although we tested a variety of protease inhibitors with different specificities for serine and cysteine proteases, only DFP inhibited receptor digestion to the $M_r \sim 52,000$ -fragment. Although the major effect of DFP is to inhibit serine proteases, commercial preparations of DFP often contain an impurity that inhibits cysteine proteases.^{21,22} The serine protease inhibitory activity of DFP is sensitive to hydrolysis, whereas the cysteine protease inhibitory activity is resistant to hydrolysis.^{21,22} Since hydrolyzed DFP did not inhibit generation of the $M_r \sim 52,000$ -receptor fragment, it appears that the protease responsible for receptor digestion belongs to the serine class of proteases. The leukemia cell protease that generates the $M_r \sim 52,000$ -receptor fragment may be different from the protease responsible for meroreceptor formation in leukemia cytosol, since the latter has been reported to be a "lysine specific" protease.^{7,10}

Mendel et al²⁸ showed that intact glucocorticoid receptors in rat thymus cytosol are converted to a $M_r \sim 52,000$ -receptor fragment when the cytosol is incubated at 3 °C for two hours. Proteolysis of the rat thymus glucocorticoid receptor is prevented by calpastatin, an inhibitor of calcium activated cysteine proteases.²⁹ Therefore it appears that receptor proteolysis in rat thymus cells is due to the activity of a calcium-activated cysteine protease.^{28,29} It seems unlikely that the same protease is responsible for digestion of the human leukemia receptor to a $M_r \sim 52,000$ -receptor fragment for the following reasons. First, idoacetamide, an inhibitor of calcium-activated cysteine proteases in mammalian cells,³⁰ did not inhibit human leukemia cell-receptor digestion. Second, the hypotonic buffer used for cytosol preparation in the present study contained EDTA, which would be expected to inactivate calcium-activated proteases. Third, under the assay conditions employed in the present study, intact receptors in rat thymus cells are stable, even when cytosol is incubated for two hours at 4 °C (Distelhorst et al, unpublished observations).

Protease inhibitors are known to affect glucocorticoid receptor binding, inactivation, degradation, and transformation *in vitro*.³¹ However, the effect of protease inhibitors on glucocorticoid receptor function *in vivo* is unknown. We are currently isolating and characterizing the serine protease from human leukemia cells that produces the $M_r \sim 52,000$ -receptor fragment. Also, we are trying to determine if this

protease affects the function or turnover of glucocorticoid receptors *in vivo*.

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In Vitro and In Vivo Effects of Deferoxamine in Neonatal Acute Leukemia

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A six week old infant with acute leukemia failed to attain remission with chemotherapy. Because we previously demonstrated that the iron chelator deferoxamine (DFO) has antiproliferative properties and modulatory effects on cell differentiation, a protocol was designed for in vitro study and for clinical use in the patient. At diagnosis, blast cells were morphologically undifferentiated, had nondiagnostic cytochemistry, showed an abnormal karyotype ($t[4;11]$), expressed markers of B cell lineage, and demonstrated $C\mu$ gene rearrangement. Tissue culture of marrow or blood cells yielded colonies of leukemic blasts. Increasing concentrations of DFO produced a dose-dependent suppression of patient's blast colony growth in vitro, and blasts within colonies showed a marked change in surface antigen expression from lymphoid to myelomonocytic

markers, became monocytic in appearance, and developed intense staining for nonspecific esterase. When DFO was given intravenously to the patient as a single agent for 48 hours, blasts no longer expressed lymphoid antigens and became strongly positive for myelomonocytic markers, identical to the in vitro findings. Intravenous DFO halted rising peripheral blood blast cell numbers and allowed a several-fold increase in normal hematopoietic progenitor colony growth. When combined with low-dose cytosine arabinoside in the treatment protocol, DFO caused striking leukemic cytoreduction. Our findings indicate that DFO has antileukemic properties by virtue of its effects on proliferation and differentiation, and they prompt further experimental and clinical studies with this agent.

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DEFEROXAMINE (DFO) is widely used therapeutically as a chelator of ferric iron in disorders of iron overload.¹ As we previously demonstrated, this drug is a potent inhibitor of DNA synthesis by human B and T lymphocytes in vitro.² Micromolar concentrations of DFO decreased intracellular levels of deoxyribonucleoside triphosphates and prevented cells from going beyond early S-phase of the cell proliferation cycle.² We also found that DFO could irreversibly inhibit proliferation and colony formation in the leukemia cell line HL-60 and could induce differentiation and DNA damage in these cells.³

Based on these properties we administered DFO for the treatment of acute leukemia in a 6-week-old patient with the disease. Studies performed on the patient's peripheral blood (PB) and bone marrow (BM) confirmed the antiproliferative properties of DFO in vitro and in vivo, as well as modulatory effects on leukemic cell differentiation.

CASE REPORT

A 6-week-old female infant was assessed for a paroxysmal harsh cough and was found to have a marked leukocytosis. She was the product of a normal full-term pregnancy, and the neonatal course was unremarkable. On physical examination she appeared well. The chest was clear to auscultation. Spleen tip and liver edge were palpable. There was no lymphadenopathy.

Her blood counts were as follows: hemoglobin 13.3 g/dL, white blood cells 94,500/ μ L with blasts comprising 52,400/ μ L, platelets 295,000/ μ L. Values for urea, creatinine, electrolytes, and uric acid levels were normal. Serum proteins, bilirubin, alkaline phosphatase and glutamic oxaloacetic transaminase levels were also normal. Cerebrospinal fluid was acellular with normal chemistry.

A bone marrow aspirate confirmed leukemic cell replacement. Blast cells were morphologically undifferentiated. Cytochemistry testing was negative for periodic-acid Schiff (PAS), Sudan-black, nonspecific esterase, and acid phosphatase. Immunologic marker studies on the diagnostic marrow cells were suggestive of B cell origin: Ia, 80%; B-4, 68%; BA-1, 14%; BA-2, 50%; B-1, negative; common acute lymphoblastic leukemia antigen (CALLA), negative; cytoplasmic IgM (cIgM), negative; surface immunoglobulin (sIg), negative. Myelomonocytic markers (MO-1, My906, My-4) T cell markers (Leu-1), and E rosette formation were all negative. Further evidence that the blast cells were derived from B cell precursors was

based on studies of immunoglobulin gene rearrangement. Following restriction enzyme digestion, a $C\mu$ probe recognized a rearrangement of one μ chain allele.⁴ Cytogenetic analysis of 26 metaphases from unstimulated marrow culture for 24 hours revealed a normal female karyotype (46XX in four cells) and a clone bearing a translocation involving the long arms of chromosomes 4 and 11 (in 22 cells). The breakpoint in chromosome 4 was in the region q21 and in chromosome 11 in the region q23.

A diagnosis of acute lymphoblastic leukemia (ALL) was made and a four-week chemotherapy treatment protocol was started: vincristine IV weekly $\times$ 4 (0.75 mg/ m^2 /dose); prednisone 2 mg/kg/day PO; daunomycin IV weekly $\times$ 3 (12.5 mg/ m^2 /dose); L-asparaginase intramuscularly (IM) 3 $\times$ weekly for nine doses (6,000 U/ m^2 /dose); and cytosine-arabioside (ARA-C) intrathecally $\times$ 1 dose, 7.5 mg total. At the conclusion of therapy peripheral blasts were still present, and a repeat marrow aspirate confirmed failure to attain remission. PB and BM blasts expressed identical immunologic markers to those demonstrated on cells from the original marrow.

With informed consent and following approval by the Human Experimentation Committee of our institution, an experimental protocol was initiated: DFO was diluted in normal saline and given by continuous infusion 10 mg/kg/h; ARA-C was given by IV push, 5 mg/kg/dose. When used together, DFO was stopped eight to 12 hours prior to ARA-C (Table 1).

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Table 1. The 20-Day Experimental Protocol Schedule*

	Deferoxamine Total Dose	Hours of Infusion	ARA-C Total Dose	WBC $\times 10^9/L$	Blasts $\times 10^9/L$
Day -2	—	—	—	16.5	3.5
Day -1	—	—	—	25.4	4.6
Day 0	—	—	—	60.9	18.2
Day 1	720	24	—	62.5	42.2
Day 2	720	24	—		
Day 3	360	12	15	63.9	40.9
Day 4	360	12	15		
Day 5	360	12	15		
Day 6	360	12	15	47.3	40.6
Day 7	—	—	15		
Day 8	—	—	15	10.9	3.1
Day 9	—	—	15		
Day 10	—	—	15	6.3	1.5
Day 11	—	—	15		
Day 12	—	—	15		
Day 13	—	—	15		
Day 14	360	12	15		
Day 15	—	—	15	4.1	0.2
Day 16	360	12	—	6.5	1.4
Day 17	1080	24	—	4.6	0.2
Day 18	720	24	—		
Day 19	720	24	—		
Day 20	720	24	—	4.5	0.1

*Deferoxamine (DFO) was given by continuous IV infusion in normal saline, 10 mg/kg/h; Cytosine-arabioside (ARA-C) was given by IV push, 5 mg/kg/dose.

MATERIALS AND METHODS

Deferoxamine. Deferoxamine (CIBA-GEIGY, Mississauga, Canada) was dissolved in iron-free RPMI culture medium (Ontario Cancer Institute, Toronto) and added to cell cultures in final concentrations of 0.75 to 2.15 $\mu\text{mol/L}$.

Preparation of cells. Heparinized BM or PB cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged ($200 \times g$, 4°C) for 20 minutes to eliminate neutrophils and red cells.

CFU-GEMM assay. The CFU-GEMM assay was performed according to Fauser and Messner^{5,6} with slight modifications. Briefly, 2×10^5 nucleated PB cells were cultured in methylcellulose with iron-free RPMI (Ontario Cancer Institute, Toronto), 30% AB serum, 5% leukocyte-conditioned medium prepared with phytohemagglutinin (PHA-LCM),⁷ human erythropoietin 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver, BC), and 5×10^5 mol/L 2-mercaptoethanol. One milliliter of the culture mixture was placed in 35-mm Petri dishes (Becton Dickinson, Oxnard, CA) and was incubated at 37°C with 5% CO_2 in air in a humidified atmosphere. All cultures were evaluated after 14 days for the number of BFU-E colonies, defined as an aggregate of more than 500 hemoglobinized cells or three or more erythroid subcolonies, CFU-C colonies of at least 40 granulocytic or macrophage cells or both, and mixed colonies containing all elements. Individual colonies were plucked from the cultures with a micropipette and analyzed for cellular composition and immunologic markers.

ALL blast colony assay. The assay used was a modification of previously described methods.^{8,9} T cells were depleted from BM or PB by standard rosetting techniques using sensitized sheep erythrocytes.¹⁰ Feeder cells for the culture were prepared from PB of normal donors by depleting the adherent fraction¹¹ and exposing it to 7,000 rads to prevent cell proliferation.

Phytohemagglutinin-conditioned medium (PHA-TCM) was prepared from the T cell PB fraction obtained from a patient with

hemochromatosis undergoing therapeutic phlebotomies as previously described.⁸

Cell culture procedure. 2×10^5 T depleted PB cells were suspended in RPMI, 10% FCS, 10% PHA-TCM, irradiated feeder cells ($10^5/\text{mL}$), and methylcellulose at a final concentration of 0.9% in 35-mm Petri dishes. The culture dishes were placed into a specialized modular incubator chamber (Billups-Rosenberg, Del Mar, CA) in a humidified atmosphere of 5% O_2 , 5% CO_2 , and balanced nitrogen. After six days of incubation, colonies were counted. A cluster of more than 20 cells was defined as a colony. Individual colonies were plucked from the culture plates and smeared on glass slides for further evaluation, using Wright's stain and nonspecific esterase staining.¹² For control, 2×10^5 irradiated feeder cells were cultured in the same assay system in each experiment to exclude their potential for colony formation.

Surface Markers

Immunocytochemical method. The cell surface antigens of air-dried smears were detected using monoclonal antibodies (MoAbs) and intestinal alkaline phosphatase, as previously described.¹³⁻¹⁵ This assay was performed to detect surface antigens on patients' PB and BM smears and colonies plucked from cultures. For colony characterization, each MoAb was tested on five to ten individual colonies that had been plucked at random from a culture plate and smeared on a glass slide. The slides were counterstained in hematoxylin for light microscopy, and the presence of surface antigen was indicated as bright red granular cellular inclusions. Morphological details were well preserved, and a high degree of correlation between morphology and surface antigen expression was observed. The intensity of staining was defined as strong (+++), moderate (++), and weak (+).

Immunofluorescence method. The cell surface phenotype was determined by indirect cell-surface fluorescence; a fluorescein-conjugated goat antimouse IgG (Fab), was used as second antibody. For each MoAb tested, 1×10^6 cells were stained and 200 cells examined. The labeling was performed on ice in the presence of 0.05% sodium azide. The percentage of cells with surface fluorescence was determined by counting cells using an inverted phase-fluorescent microscope.

Monoclonal antibodies. The following MoAbs were used in these studies: anti-OK1a (Ortho Diagnostic Systems, Raritan, NJ) to detect HLA-D framework antigen; anti-MY-7, anti-Mol and anti-MY-4 (Coulter Immunology, Hialeah, FL) to identify myelomonocytic markers; anti-BA1, anti-BA2, anti-B1 and anti-B4 (Becton Dickinson, Oxnard, CA) to recognize cells of the B cell lineage; anti-J5 (Coulter Immunology) to recognize cells bearing common ALL (CALLA) antigen; and anti-Leu-1 and anti-Leu-4 (Coulter Immunology) to recognize cells of the T cell lineage.

RESULTS

In Vitro

Blast colony assay. Prior to therapy with DFO, patient's PB blast cells were cultured using the ALL blast colony assay; they yielded a mean of 700 colonies/ 2×10^5 cells plated. Individual colonies were plucked from the culture plates and were examined for cellular composition. Morphologically they were undifferentiated blast cells identical to the appearance of marrow cells at diagnosis. The cells were negative for nonspecific esterase by cytochemistry. Surface antigen studies of PB cells confirmed lymphoblastic markers and absent myelomonocytic markers (Table 2).

Antiproliferative effect of DFO. As shown in Fig 1, when increasing concentrations of DFO were added to

Table 2. Surface Marker Analysis of Leukemia Cell Colonies (% Positive Cells)

	Control	2.5 μ mol Deferoxamine
Ia	80% (+++)	80% (+++)
CALLA	0%	0%
B4	80% (+++)	30% (+++)
Mol	0%	>90% (+++)
MY-4	0%	60% (++)
Leu-1	0%	0%
Leu-4	0%	0%

PB leukemia cell colonies were plucked at random from cultures containing 2.5 μ mol deferoxamine and were compared to cultures without deferoxamine using the immunocytochemical assay.

cultures of patient's PB blast cells, there was a dose-dependent suppression of blast colony growth. Significant decline in blast colonies was observed with DFO concentrations greater than 1.25 μ mol, and with 2.15 μ mol DFO, a 70% reduction in blast colonies was observed. In a control study of normal marrow obtained from a healthy volunteer, DFO also produced a dose-dependent suppression of normal hematopoietic colonies, CFU-C, CFU-E, and BFU-E. However, 10 μ mol DFO was necessary to achieve a 70% reduction in colony counts.

DFO and blast cell differentiation. Individual blast colonies from the culture plates containing DFO 2.5 μ mol were characterized (Table 2). Compared to colonies grown without exposure to DFO, a marked change in cell surface antigen expression was observed. Blast colonies from the DFO plates strongly expressed the myelomonocytic markers Mol and MY-4 as determined by immunocytochemistry, and numbers of B-4 positive cells were markedly reduced. Moreover, individual cells from colonies exposed to DFO were changed in morphology, appearing monocytic, and stained intensely for nonspecific esterase that could be inhibited by fluoride.

In Vivo

DFO was administered therapeutically according to the protocol outlined in Table 1.

DFO and blast cell differentiation. After 48 hours of continuous infusion of DFO as a single agent, striking changes in blast cell surface markers were detected immunocytochemically (Table 3). Prior to treatment no blasts expressed Mol antigen, and only 8% weakly expressed MY-4. After 48 hours cells expressing these two antigens, Mol and MY-4, were detected in peripheral blood blast cells. Concomitantly with DFO therapy there was a sharp decline

Table 3. Surface Marker Analysis Pre- and Post-Therapy (% Positive Cells)

	Before Deferoxamine Treatment	After Deferoxamine Treatment
Ia	80% (+++)	80% (+++)
CALLA	2% (+)	0% (+)
B4	37% (++)	0%
Mol	0%	40% (+++)
MY-4	8% (+)	60% (+++)
Leu-1	0%	0%
Leu-4	0%	0%

Immunologic surface markers were determined before and after 48 hours of deferoxamine treatment on PB smears using the immunocytochemical method.

from 37% to 0% of cells bearing B4 antigen. These data were confirmed by immunofluorescence studies.

At the conclusion of the 20-day treatment protocol, a marrow aspirate showed 60% blasts that were negative for PAS, Sudan-black, and acid phosphatase. In contrast to the marrow aspirate prior to DFO therapy, 60% of the blasts were positive for nonspecific esterase, and staining could be inhibited by fluoride. Surface marker studies performed concurrently demonstrated expression of myelomonocytic antigens that were not detected in marrow cells prior to treatment (Table 4).

Antiproliferative effect of DFO. The WBC and blast number had increased several-fold in the three days prior to DFO treatment (Table 1). After the first 48 hours of DFO therapy, the counts reached a plateau. Significant decline of WBC and blast numbers was observed on day 8, that is, six days following combination DFO-ARA-C therapy. By day 15, PB blasts almost completely disappeared.

Several CFU-GEMM assays were performed on PB during the first 48 hours of DFO treatment (Table 5). Impressive increases in normal hematopoietic progenitor colony growth were documented. Erythroid and granulocytic progenitor colony formation increased several-fold over pre-DFO treatment colony numbers.

Table 4. Surface Marker Analysis Before and at Conclusion of 20-Day Treatment (% Positive Cells)

	Before Deferoxamine Treatment	After Deferoxamine Treatment
Ia	80%	75%
slg	10%	0%
BA-1	14%	0%
BA-2	50%	0%
B1	Negative	Negative
B4	68%	10%
CALLA	Negative	Negative
E rosettes	Negative	Negative
Leu-1	Negative	Negative
MY-7	0%	20%
Mol	0%	20%
MY-4	10%	20%

Immunologic surface markers were determined by immunofluorescence before and at the conclusion of the 20-day treatment course on bone marrow cells.

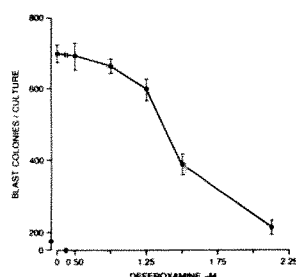


Fig 1. The effect of increasing concentrations of deferoxamine on patient's PB ALL blast colony numbers.

Table 5. Numbers of CFU-GEMM Colonies Pre- and Post-Therapy

Colony Type	Before Deferoxamine Treatment	After Deferoxamine Treatment
BFU-E	9 ± 1	113 ± 14
CFU-C	45 ± 6	126 ± 17
Mixed	1 ± 1	5 ± 2

CFU-GEMM colony culture of patient's PB was performed before and after 48 hours deferoxamine treatment.

DISCUSSION

The infant who was studied had leukemia that was unresponsive to conventional chemotherapy. At the conclusion of induction therapy, blasts were still present in PB and in marrow, and when chemotherapy was stopped, PB blast counts increased several-fold in three days.

The blasts were undifferentiated according to morphological and cytochemical criteria and expressed antigens of B lineage at diagnosis. Further confirmation of their origin from B cells was by analysis of DNA from the leukemic cells that revealed rearrangement of one of the immunoglobulin heavy-chain genes. The presence of an abnormal karyotype, t(4;11), as found on cytogenetic analysis of BM cells, has been previously reported in neonatal ALL.¹⁶ This form of leukemia is characterized by PB leukocytosis, blast cells with an undifferentiated appearance, and poor prognosis.¹⁷ Although the cytogenetic abnormality has been found in ALL,¹⁸ it has also been seen in cases of acute leukemia of myelomonocytic lineage.¹⁷

We were prompted to use DFO in an experimental treatment protocol because of our previous finding that the agent was a reversible early S-phase inhibitor of human lymphocyte proliferation.² Although widely used as a chelator of ferric iron in disorders of iron overload, we demonstrated that the drug is a potent inhibitor of DNA synthesis by B and T lymphocytes in vitro, likely by impairing the activity of the iron-containing enzyme ribonucleotide reductase through a chelating mechanism.² More recently we have shown that DFO can induce monocyte-macrophage cell differentiation in the human promyelocytic leukemic cell line, HL-60, as judged by altered expression of cell surface antigens, nonspecific esterase activity, and morphological changes combined with irreversible inhibition of colony formation.³ The mechanism appeared to be related to inhibition of replicative DNA synthesis or due to an increase in number of single-strand DNA breaks. Because DFO has antiproliferative properties as well as modulatory effects on cell differentiation, it may be potentially useful for experimental and therapeutic applications.

In vitro we were able to demonstrate the antiproliferative effects of DFO on leukemic cell growth from our patient using a blast colony assay. Because the plating efficiency of PB blast cells was very high, clear-cut suppression of colony growth by DFO could be readily demonstrated. The DFO-induced change in blast cell surface antigen expression from lymphoid to myelomonocytic was striking and confirmed the modulatory effect of DFO on cell differentiation.

The in vitro changes correlated closely with the findings during the first 48 hours of continuous infusion of DFO when used as a single agent. The PB WBC and blast counts, which

had risen dramatically for three days prior to DFO therapy, leveled off 48 hours after initiation of DFO infusion, suggesting an in vivo antiproliferative effect. Moreover, surface marker antigen changes were observed on the blast cells that were identical to those seen after DFO treatment in vitro. There was convincing evidence for expression of myelomonocytic antigens and concurrent disappearance of lymphoid markers. The possibility that during the 48 hours of DFO treatment a change in PB cell distribution was responsible for the change in surface markers was unlikely, since the blast count remained stable during this period. With the HL-60 cell line, Boyd and Metcalf showed that the differentiation process is associated with irreversible loss of proliferative ability, inhibition of DNA synthesis, and G₀/G₁ cell cycle arrest.¹⁹ Since identical changes in the same cell line were induced by DFO,³ this could be the mechanism by which DFO affected our patient's leukemic cells. Although the loss of proliferative ability was suggested by our data, it was not definitively proven using the colony assay technique.

The cellular lineage of the blasts is an important aspect of the case because there was a change in differentiation markers with therapy. It is unlikely that two separate leukemic populations were responsible for the dual marker findings because the abnormal karyotype found at diagnosis was still detected at the conclusion of therapy, suggesting persistence of the same leukemic clone. There have been reports of conversion with chemotherapy of cases of non-T ALL to myelomonocytic leukemia²⁰ and from T-lymphoblastic leukemia to promyelocytic leukemia.²¹ In the latter case it was shown that the leukemia was actually derived from a stem cell.²¹ Although we have observed the pattern of immunoglobulin heavy-chain gene rearrangement only in cases of ALL,⁴ the abnormal karyotype found in our patient's blast cells has been seen in both ALL and in ANLL.^{16-18,20} Moreover, the 4;11 translocation is remarkable for its association with biphenotypic expression of differentiation markers.²²⁻²⁴ In our case it is possible that the blasts showed "lineage infidelity"²⁵ and expressed markers from two lineages at different times. With regard to the mechanism, one may speculate that the 4;11 syndrome is a stem cell disorder that can differentiate along one lineage given a set of growth factors and in another lineage given others.

A low plating efficiency of normal pluripotent hematopoietic precursors in acute leukemia, as demonstrated in our patient, has been previously described.^{26,27} The significantly higher plating efficiency observed using the CFU-GEMM assay of erythroid as well as granulocyte-macrophage elements from our patient's PB following DFO treatment occurred in spite of persistently high numbers of PB blast cells. It is unlikely that a high percentage of the blast-like cells were actually normal hematopoietic progenitors because of their increased numbers and morphological features. It is possible that DFO in vivo induced a change in the leukemic blasts allowing their differentiation toward the erythroid as well as the granulocyte-macrophage lineage as previously described in Ph⁺-positive chronic myelogenous leukemia.^{28,29} Another interpretation is that DFO treatment in some manner permitted the proliferation of normal hema-

topoietic progenitors that were suppressed by the leukemic cells as described by Broxmeyer et al.^{30,31}

The rationale for using ARA-C with DFO in the treatment protocol was based on the hypothesis that if DFO could reversibly block leukemic cells in S-phase, after removal of DFO, ARA-C should destroy the cells progressing through S-phase because its cytotoxic action is S-phase-dependent.

From the available data it is still unclear whether the apparent synergism between DFO and ARA-C is attributed to S-phase synchronization or to the differentiating action of DFO. The marked clinical reduction in PB blast numbers, the changes in leukemic cell marker expression, and the in vitro and in vivo antiproliferative properties prompt further experimental and clinical studies with DFO.

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Activation of Neutrophil Superoxide Production by Concanavalin A Can Occur at Low Levels of Intracellular Ionized Calcium

By John C. Whitin, Kazuhiko Takahashi, and Harvey J. Cohen

The effect of concanavalin A (Con A) on the concentration of ionized intracellular calcium ($[Ca_i^{++}]$) in human granulocytes (PMN) was monitored using the fluorescent calcium indicator and chelator, Quin2. The addition of Con A to PMN resulted in a rise in $[Ca_i^{++}]$ that was markedly enhanced by the presence of Ca^{++} in the external buffer. The onset of the increment in $[Ca_i^{++}]$ preceded the onset of O_2^- production. The rise in $[Ca_i^{++}]$ induced by Con A is not transient, with a new, higher steady-state level of $[Ca_i^{++}]$ being attained within five minutes. The addition of α -methylmannoside (α -MM) one minute after Con A resulted in the return of $[Ca_i^{++}]$ to the original baseline level and the cessation of O_2^- production. The addition of a second stimulus (such as arachidonic acid) to these cells resulted in a second increment in $[Ca_i^{++}]$ and the return of O_2^- production. Thus the rise in $[Ca_i^{++}]$ induced by Con A is tightly coupled to the activation, inactivation, and reactivation of the O_2^- generating system by Con A. Further experiments were undertaken to assess the possible requirement for the rise in $[Ca_i^{++}]$ in the activation of PMN by Con A. PMN could be depleted of Ca_i^{++} by loading with Quin2 in the absence of extracellular Ca^{++} . These Ca^{++} -depleted PMN can be induced to produce O_2^- after treat-

ment with PMA but not with Con A. The addition of Ca^{++} to Ca^{++} -depleted PMN results in a return of $[Ca_i^{++}]$ to the normal resting level of Ca^{++} -replete PMN. The time required to return to baseline is a function of the concentration of intracellular Quin2. The addition of Ca^{++} to Ca^{++} -depleted, Con A-treated PMN results in the elevation of $[Ca_i^{++}]$ and the production of O_2^- . Over a tenfold range of intracellular Quin2 concentration, the onset of O_2^- production always occurred at $[Ca_i^{++}]$ that were less than the normal resting level. Thus, activation of the O_2^- generating system by Con A can occur at $[Ca_i^{++}]$ which are much lower than the incremental level induced by Con A in Ca^{++} -replete PMN. Supporting this is the observation that only a very small increment in $[Ca_i^{++}]$ is induced by Con A in PMN cytoplasts, even though Con A could induce O_2^- production in the Quin2-loaded cytoplasts. While a Con A-induced increment in $[Ca_i^{++}]$ is a result of stimulus-response coupling and correlates with the activation of the PMN O_2^- generating system, these observations raise doubts about the necessity of a large change in $[Ca_i^{++}]$ in the activation of the O_2^- generating system.

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HUMAN NEUTROPHILS (PMN) respond to various stimuli with the initiation of O_2^- production, secretion of granule contents, chemotaxis, aggregation, or phagocytosis.¹⁻³ Other responses of PMN to stimulation include changes in membrane potential,^{4,5} phosphatidyl inositol metabolism,⁶ protein phosphorylation,^{7,8} membrane-bound calcium,^{9,10} and the concentration of intracellular ionized calcium ($[Ca_i^{++}]$).^{11,12} These latter responses have received much attention because of their potential causal involvement in the activation of PMN.

The use of concanavalin A (Con A) as a stimulant of PMN responses is useful because of the ability of α -methylmannoside (α -MM) to reversibly terminate activation by Con A.¹³⁻¹⁵ In this way the relationships between different PMN responses can be analyzed. For example, when Con A-treated PMN are treated with α -MM, O_2^- production ceases, while granule secretion does not.¹⁵ These PMN are primed for a challenge by an additional stimulus: lag times prior to O_2^- production are shorter, their membrane potential remains depolarized, and they have a diminished requirement for extracellular Ca^{++} for reactivation of O_2^- production by Con A.

Using the trapped Ca^{++} indicator Quin2, we found that Con A induces a sustained rise in Ca_i^{++} that is reversed by the subsequent addition of α -MM. The addition of a second stimulus then results in a second increment of Ca_i^{++} , thus demonstrating that Ca_i^{++} increases every time PMN are activated. We also present data that show that activation of PMN by Con A can occur at levels of Ca_i^{++} that are below the levels obtained after treatment with a stimulus.

MATERIALS AND METHODS

Arachidonic acid, bovine serum albumin, cytochrome c type VI, ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetra-acetic acid

(EGTA), Ficoll 70, HEPES, lysophosphatidylcholine (LPC), α -MM, p-nitrophenyl β -D-glucuronide, p-nitrophenylphosphate, superoxide dismutase, and triton X-100 were purchased from Sigma Chemical, St Louis; phorbol myristate acetate (PMA) from Consolidated Midland, Brewster, NY; cytochalasin B from Aldrich Chemicals, Milwaukee, WI; Ficoll-Paque and Dextran T-500 from Pharmacia Fine Chemicals, Piscataway, NJ; and the fluorescent calcium indicator Quin2 and Quin2-tetra-acetoxymethyl ester (Quin2 A/M) from Behring Diagnostics, La Jolla, CA. Stock solutions were prepared as follows: Con A at 2 mg/mL in distilled H_2O ; cytochalasin B at 1.0 mg/mL in dimethyl sulfoxide (DMSO); PMA at 2 mg/mL in DMSO and diluted to 20 μ g/mL in buffer for use; and LPC at 2 mg/mL in ethanol. Arachidonic acid was diluted to 2.5 mg/mL in 25% vol/vol ethanol and clarified with 0.1 N NaOH. Quin2 A/M was dissolved at 10 mmol/L in DMSO.

Human peripheral blood PMN were isolated utilizing Dextran T-500 and Ficoll-Paque sedimentation followed by hypotonic lysis of contaminating erythrocytes as previously described.^{16,17} PMN were finally resuspended in phosphate buffered saline (PBS; 135 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L sodium phosphate buffer, pH 7.40) at 5×10^7 /mL and kept in ice.

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PMN cytoplasts were prepared essentially as previously described.¹⁸ PMN at 1×10^8 /mL were incubated in 12.5% (wt/vol) Ficoll 70 containing 20 μ mol/L cytochalasin B for five minutes at 37 °C. These PMN were then centrifuged at $81,000 \times g$ in a Beckman SW-27 rotor at 33 °C for 30 minutes over a prewarmed discontinuous Ficoll gradient of 18% and 25% (wt/vol) Ficoll 70 containing 20 μ mol/L cytochalasin B. The cytoplasts were harvested from the interface between the 12.5% and 18% Ficoll layers. Control PMN (treated at 33 °C in 12.5% Ficoll containing 20 μ mol/L cytochalasin B) and cytoplasts were then washed three times in PBS containing 5 mmol/L glucose and 0.5% BSA and one time in PBS alone before counting and marker analysis. The yield of cytoplasts on a cellular basis was 71% of the PMN layered on the gradient. Depletion of granules in cytoplasts was assessed by measuring the content of β -glucuronidase as previously described.¹⁹ The content of alkaline phosphatase was used as a marker of plasma membrane and was assayed as previously described.¹⁸ Cytoplast β -glucuronidase content was 2% of PMN when normalized per cell and 4% of PMN when normalized per unit of alkaline phosphatase.

O_2^- production was monitored continuously as the superoxide dismutase-inhibitable reduction of cytochrome c as previously described.²⁰ The buffer was Krebs-Ringer Phosphate (KRP; PBS plus 1 mmol/L $CaCl_2$ and 1.3 mmol/L $MgSO_4$) unless stated otherwise. Cytochalasin B (10 μ mol/L) was routinely added to all assays of PMN function, as we find that its presence enhances Con A-induced O_2^- production 20-fold.

Human PMN were loaded with Quin2 A/M essentially as described previously.^{11,21} PMN at 5×10^7 /mL in PBS containing 1.0 mmol/L $CaCl_2$, 5 mmol/L glucose and 0.5% bovine serum albumin were incubated with 50 μ mol/L Quin2 A/M for ten minutes at 37 °C, after which they were diluted to 1×10^7 /mL with the same buffer and the incubation continued for an additional 20 minutes. At that time the PMN were diluted with one volume of cold PBS, centrifuged at 4 °C, washed one time with cold PBS, and resuspended to 5×10^7 /mL PBS. In some experiments the PMN were depleted of Ca_i^{++} by incubation with varying concentrations of Quin2 A/M in the absence of Ca^{++} as previously described.^{22,23} Responses of these PMN were monitored in HEPES-buffered saline (HBS; 135 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, pH 7.40). Where indicated 1 mmol/L $CaCl_2$ was also added.

The fluorescence of Quin2-loaded PMN was monitored essentially as described previously.^{11,21} PMN (5×10^6 in 1.0 mL buffer)

were stirred in the thermostatted (37 °C) sample compartment of a Perkin Elmer 650-10S spectrofluorometer. The excitation wavelength was set at 339 nm (band pass slit of 4 nm), and the emission wavelength was set at 492 nm (band pass slit of 10 nm). The $[Ca_i^{++}]$ was calculated from the maximum fluorescence obtained in PMN lysed with 10 μ g/mL LPC in the presence of 1.0 mmol/L Ca^{++} and the minimum fluorescence of LPC-lysed PMN in the presence of 10 mmol/L EGTA as described previously.²¹ A standard curve of the fluorescence of known amounts of Quin2 in Ca^{++} -containing suspensions of LPC-lysed PMN was also prepared. The fluorescence of lysed Quin2-loaded PMN in the presence of 1.0 mmol/L Ca^{++} was then determined and compared with the standard curve.

PMN and cytoplasts were loaded with chlortetracycline as described by Smolen et al.⁹ PMN at 1×10^7 /mL and cytoplasts at 2.2×10^7 /mL were loaded with 50 μ mol/L chlortetracycline for 20 minutes at 37 °C, after which they were washed by centrifugation and resuspended in PBS. Fluorescence tracings were performed at an excitation wavelength of 380 nm (slit of 6 nm) and an emission wavelength of 560 nm (slit of 6 nm).

RESULTS

Effect of activation of PMN on $[Ca_i^{++}]$. As many reports have recently shown,^{11,12,24} Quin2-loaded PMN can be used to monitor changes in $[Ca_i^{++}]$ induced by various stimuli. Figure 1A shows that Quin2-loaded PMN, pretreated with cytochalasin B (necessary for maximum ConA induced O_2^- production), respond to Con A with an increase in $[Ca_i^{++}]$. The onset of the increment in $[Ca_i^{++}]$ occurs within 15 seconds and precedes the onset of O_2^- production at 30 to 50 seconds. For 12 blood donors, the resting $[Ca_i^{++}]$ for PMN under these conditions is 110 ± 18 nmol/L (mean $\pm$ SD) while that of the peak value of $[Ca_i^{++}]$ induced by Con A is 547 ± 174 nmol/L. After the peak has been achieved there is a subsequent decline in $[Ca_i^{++}]$, which reaches a new steady-state level (a mean of 259 ± 19 nmol/L, $n = 6$) in approximately three minutes. Essentially the same phenomenon occurs when Quin2-loaded PMN are treated with 12.5 μ g/mL arachidonic acid. Figure 1B shows that Quin2-loaded PMN treated with arachidonic acid also undergo a sustained increase in $[Ca_i^{++}]$ that precedes the onset of O_2^- production.

Effect of inactivation of stimulated PMN on $[Ca_i^{++}]$. If the Quin2-loaded PMN are instead treated with 25 mmol/L α -MM one minute after treatment with Con A (at the peak of the $[Ca_i^{++}]$ transient, Fig 2A) the Con A-induced increment in $[Ca_i^{++}]$ is completely reversed and $[Ca_i^{++}]$ returns to its initial, resting level. Treatment of Con A-stimulated PMN with α -MM in this manner results in the cessation of O_2^- production. Thus treatment of PMN with Con A results in a reversible increase in $[Ca_i^{++}]$. In addition, the fluorescence response to Con A appears to be biphasic. The sustained increase in $[Ca_i^{++}]$ induced by Con A is not an artifact due to the Ca^{++} -buffering capacity of Quin2, since the same results are obtained if the PMN are first incubated with Con A and are then loaded with Quin2 (data not shown). In addition, PMN that are treated with Con A followed by α -MM one minute later and are then loaded with Quin2 display the normal resting $[Ca_i^{++}]$. While the time course of changes in $[Ca_i^{++}]$ may be influenced by the buffering capacity of Quin2 as described for FMLP-treated PMN,²⁵ the phenomena are the same whether Quin2-loaded

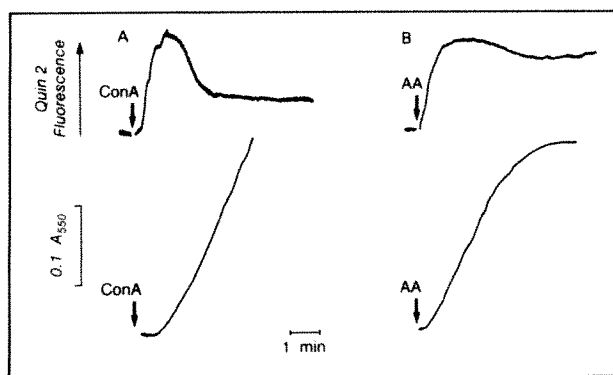


Fig 1. Effect of Con A and arachidonic acid on $[Ca_i^{++}]$ and O_2^- production. Fluorescence tracing of 5×10^6 PMN/mL KRP that had been loaded with 50 μ mol/L Quin2 A/M as described in "Methods" (upper tracings) and continuous measurement of O_2^- production (lower tracings). (A) The addition of 100 μ g/mL Con A (at the arrow) in the presence of 10 μ mol/L cytochalasin B. (B) The addition of 12.5 μ g/mL arachidonic acid (at the arrow) in the presence of 10 μ mol/L cytochalasin B.

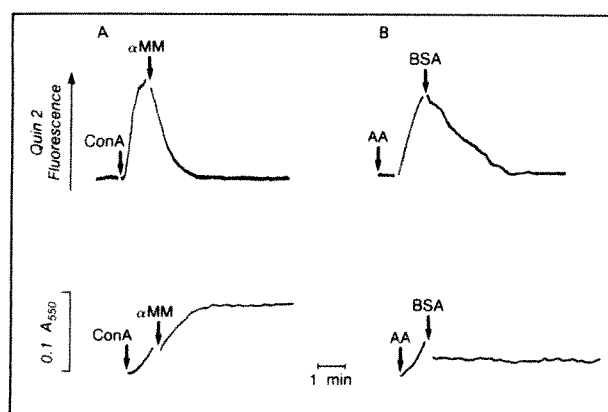


Fig 2. Effect of removal of stimuli on $[Ca_i^{++}]$ and O_2^- production. Fluorescence tracings of Quin2-loaded PMN and O_2^- production as in Fig 1. (A) The addition of 25 mmol/L α -MM (at the second arrow) to Con A-treated PMN. (B) The addition of arachidonic acid (first arrow) followed by the addition of 1.35 mg/mL fatty acid-free bovine serum albumin (BSA) (second arrow).

or control PMN are treated with Con A. In a similar manner, Quin2-loaded PMN treated with arachidonic acid followed by fatty acid-free bovine serum albumin at one minute cease producing O_2^- (as shown in previous reports²⁶⁻²⁸) and undergo a reversal of their increase in $[Ca_i^{++}]$ to their original resting level (Fig 2B).

Effect of reactivation of PMN on $[Ca_i^{++}]$. Previously activated PMN can be reactivated to produce O_2^- by the subsequent addition of another stimulus.^{14,15} As can be seen in Fig 3A, when arachidonic acid is added to PMN previously treated with Con A followed by α -MM, the $[Ca_i^{++}]$ increment is the same as when resting PMN are treated with arachidonic acid (Fig 1B). Figure 3A also shows that arachidonic acid can induce O_2^- production in PMN previously treated with Con A followed by α -MM. PMN that have been activated with arachidonic acid and inactivated with fatty

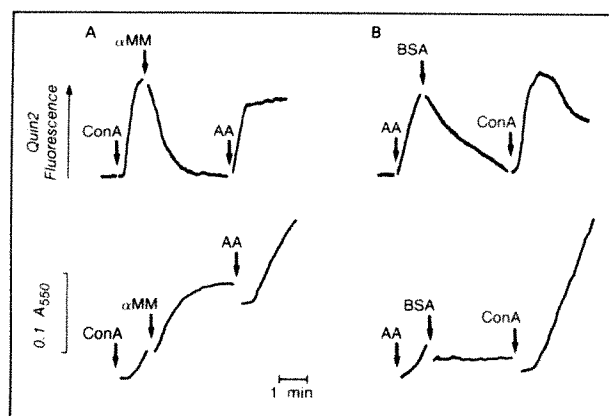


Fig 3. Effect of second stimuli on $[Ca_i^{++}]$ and O_2^- production. Fluorescence tracings of Quin2-loaded PMN and O_2^- production in the presence of 10 μ mol/L cytochalasin B as in Fig 1. (A) The addition of Con A (first arrow) followed by α -MM (second arrow) followed by arachidonic acid (third arrow). (B) The addition of arachidonic acid (first arrow) followed by BSA (second arrow) followed by Con A (third arrow).

acid-free bovine serum albumin can also be reactivated by the addition of an additional stimulus such as Con A (Fig 3B). The addition of Con A results in an increment in $[Ca_i^{++}]$ and the resumption of O_2^- production. Therefore, for both Con A and arachidonic acid, the increment in $[Ca_i^{++}]$ occurs when PMN are either activated or reactivated to produce O_2^- (Fig 1 and 3), while for both of these stimuli inactivation of the O_2^- generating system by removal of the stimulus also results in the return of $[Ca_i^{++}]$ to the original resting level (Fig 2).

Effect of Con A on cytoplasmic $[Ca_i^{++}]$. The same type of experiment using PMN cytoplasts was performed. Control PMN (5×10^7 /mL, heated to 33 °C for 30 minutes in 12.5% Ficoll/20 μ mol/L cytochalasin B) and cytoplasts (approximately 1.1×10^8 /mL, having the same alkaline phosphatase content as control PMN) were loaded with Quin2 A/M. The resting $[Ca_i^{++}]$ of cytoplasts was 66 ± 3 nmol/L compared to the $[Ca_i^{++}]$ of 151 ± 20 nmol/L for these control PMN (Fig 4). When these Quin2-loaded cytoplasts were treated with Con A, there was a very small increment in $[Ca_i^{++}]$ to 78 nmol/L over several minutes. In contrast, the $[Ca_i^{++}]$ of these control PMN increased to 302 nmol/L after treatment with Con A. Despite the extremely small increment in $[Ca_i^{++}]$ induced by Con A, the Quin2-loaded cytoplasts generated O_2^- at approximately 50% of the rate of control PMN (normalized per unit of alkaline phosphatase, Table 1), with lag times for Con A O_2^- production of approximately 100 seconds. This very small increment in $[Ca_i^{++}]$ of cytoplasts induced by Con A was both inhibitable and reversible with α -MM as was O_2^- production (data not shown). This would indicate that PMN cytoplasts can be activated by Con A to produce O_2^- at levels of $[Ca_i^{++}]$, which are significantly lower than even the normal resting level of PMN. The release of membrane-bound calcium (as measured by following changes in fluorescence of chlortetracycline-loaded cells) was also compared for control PMN and cytoplasts. As shown in Fig 5, the addition of Con A to both PMN and cytoplasts results in a loss of chlortetracycline fluorescence, indicative of a decrease in membrane-associated calcium. In contrast to the Quin2 tracings, the time course of fluorescence change is smaller for both PMN

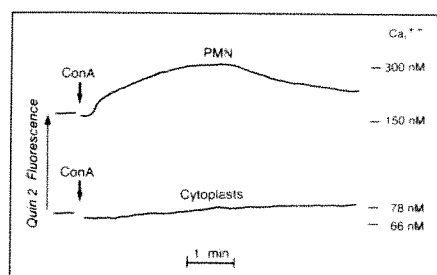


Fig 4. Fluorescence tracings of cells loaded with 50 μ mol/L Quin2 A/M in KRP. The addition of 100 μ g/mL Con A to 1.1×10^7 cytoplasts/mL in the presence of 10 μ mol/L cytochalasin B. For comparison, the addition of Con A to 5×10^6 control PMN/mL in the presence of 10 μ mol/L cytochalasin B is also shown. The resting and stimulated $[Ca_i^{++}]$ are indicated for both cell types. Comparable results were obtained in each of three separate cytoplast preparations.

Table 1. O_2^- Production by Quin2-Loaded PMN and Cytoplasts

Cells	Stimulus	nmol O_2^- /min/alkaline Phosphatase
PMN	Con A	8.8
PMN	PMA	3.7
PMN	Arachidonic acid	3.5
Cytoplasts	Con A	3.7
Cytoplasts	PMA	1.6
Cytoplasts	Arachidonic acid	2.4

Cytoplasts and control PMN were prepared as described in the text, after which they were loaded with 50 μ mol/L Quin2 A/M. Rates of O_2^- production were then determined for stimulation by 100 μ g/mL Con A, 1 μ g/mL PMA, and 12.5 μ g/mL arachidonic acid in the presence of 10 μ mol/L cytochalasin B. Results are normalized to equivalent amounts of the plasma membrane marker alkaline phosphatase, reported as arbitrary units. Shown are the averages of duplicate determinations (which did not differ by more than 20%) of an experiment performed two times with comparable results.

and cytoplasts and more closely correlates with the lag time for O_2^- production. The ability of PMN to be activated by Con A at low $[Ca_i^{++}]$ was tested by the following experiments.

Studies using Ca^{++} depleted PMN. PMN loaded with Quin2 A/M in the absence of extracellular Ca^{++} can become depleted of $[Ca_i^{++}]$, as previously reported for macrophages and PMN.^{22,23} Therefore PMN were incubated with 10 to 100 μ mol/L Quin2 A/M in the absence of extracellular Ca^{++} , after which they were washed in PBS and resuspended in PBS as usual. The intracellular content of Quin2 was determined as described in the methods section. For these

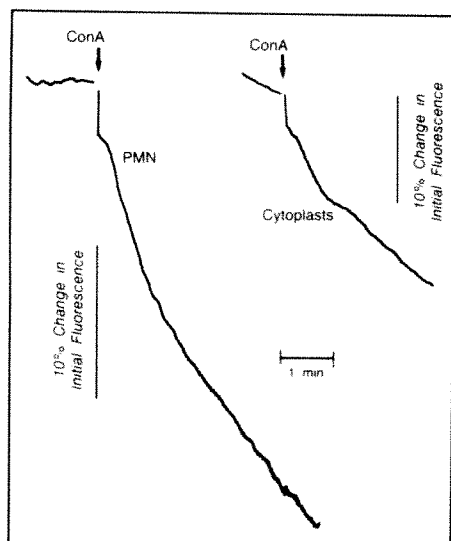


Fig 5. Fluorescence tracings of chlortetracycline-loaded PMN and cytoplasts. PMN and cytoplasts were loaded with chlortetracycline as described in "Methods." Tracings of fluorescence changes induced by 100 μ g/mL Con A in the presence of 10 μ mol/L cytochalasin B were then obtained for 5×10^6 PMN/mL and 1.1×10^7 mL KRP. A 10% change in initial fluorescence is indicated for each sample, and the absolute fluorescence scale is the same for both tracings. The two tracings are zero-offset by different amounts. Comparable results were obtained in two cytoplast preparations.

PMN the content of Quin2 ranged from 0.2 to 2.0 nmol/ 10^6 cells.

The exact value of $[Ca_i^{++}]$ of Ca^{++} -depleted PMN is difficult to calculate. The fluorescence in PBS of PMN depleted of Ca^{++} with Quin2 was the same as the fluorescence of Quin2-loaded PMN that had been permeabilized with LPC in the presence of 10 mmol/L EGTA. As measured with a Ca^{++} electrode,²⁹ the Ca^{++} concentration of this buffer in the presence of EGTA appears to be less than 20 nmol/L, but the exact value is not known.

In the absence of extracellular Ca^{++} , these PMN were unable to generate O_2^- in response to Con A. However, these PMN were able to generate O_2^- in response to PMA (Table 2), indicating that the PMN were not damaged by depletion of $[Ca_i^{++}]$ as shown previously.²³

The addition of Con A to Ca^{++} -depleted PMN in the absence of additional Ca^{++} does not result in an increase in the indicated $[Ca_i^{++}]$ (Fig 6A). If Ca^{++} is then added to these PMN in the presence of Con A, $[Ca_i^{++}]$ rises and reaches a new steady state level (Fig 6B). The $[Ca_i^{++}]$ of these PMN does not increase further when Con A is added again. In contrast, Ca^{++} -depleted PMN that have been treated first with Ca^{++} followed by Con A do respond with the characteristic stimulus-induced increase in $[Ca_i^{++}]$ (Fig 6C). The time course for the increase in Quin2 fluorescence was a function of the amount of Quin2 found within the PMN (tracings not shown). At all of the concentrations of intracellular Quin2 tested, $[Ca_i^{++}]$ finally returns to the resting level of normal PMN.

These Ca^{++} -depleted PMN were able to generate O_2^- in response to Con A after repletion of $[Ca_i^{++}]$. Figure 7 shows the ability of these Ca^{++} -depleted PMN to generate O_2^- in response to Con A following a ten-minute incubation with Ca^{++} . When $[Ca_i^{++}]$ of these Ca^{++} -depleted PMN is allowed to return to the normal resting level prior to activation with Con A (open symbols in Fig 7), both the lag time prior to O_2^- production and the rate of O_2^- production are close to control values. Thus these PMN can be reversibly depleted of Ca^{++} and can regain their ability to produce O_2^- in response to Con A.

Table 2. Effect of Ca-Depletion on Ability of PMN to Generate O_2^-

Quin2 A/M Incubation	O_2^- Assay	Stimulus	nmol O_2^- /min/ 10^6 PMN
- Ca^{++}	- Ca^{++}	Con A	0
- Ca^{++}	+ Ca^{++}	Con A	3.5 ± 1.7
+ Ca^{++}	- Ca^{++}	Con A	0.1 ± 0.1
+ Ca^{++}	+ Ca^{++}	Con A	6.6 ± 0.4
- Ca^{++}	- Ca^{++}	PMA	6.7 ± 0.8
- Ca^{++}	+ Ca^{++}	PMA	6.4 ± 0.1
+ Ca^{++}	- Ca^{++}	PMA	6.5 ± 0.8
+ Ca^{++}	+ Ca^{++}	PMA	6.4 ± 1.0

PMN were loaded with 50 μ mol/L Quin2 A/M in the presence or absence of 1 mmol/L $CaCl_2$ as indicated. O_2^- production was then measured in the presence or absence of 1 mmol/L $CaCl_2$ and 10 μ mol/L cytochalasin B after a five-minute incubation at 37 $^\circ$ C as indicated. The concentrations of stimuli were: 100 μ g/mL Con A or 1 μ g/mL PMA. Shown are the triplicate values of one experiment (mean $\pm$ SD), repeated two times with comparable results.

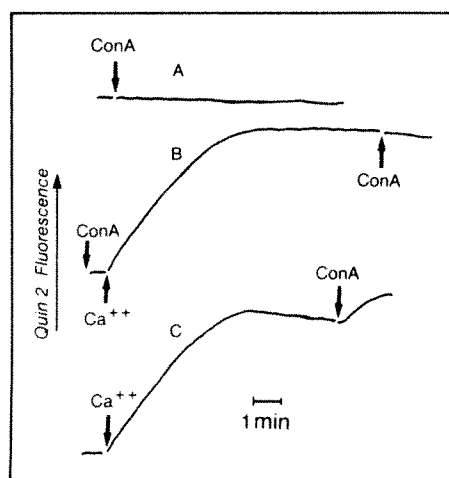


Fig 6. Fluorescence tracings of Ca^{++} -depleted PMN loaded with 50 $\mu\text{mol/L}$ Quin2 A/M in the absence of Ca^{++} . (A) The addition of 100 $\mu\text{g/mL}$ Con A to Ca^{++} -depleted PMN in HBS. (B) The addition of 100 $\mu\text{g/mL}$ Con A to Ca^{++} -depleted PMN in HBS followed by the addition of 1 mmol/L CaCl_2 , followed by a second addition of 100 $\mu\text{g/mL}$ Con A. (C) The addition of 1 mmol/L CaCl_2 to Ca^{++} -depleted PMN in HBS. Con A is then added after stabilization of fluorescence. Cytochalasin B (10 $\mu\text{mol/L}$) was present in all samples.

The next study was designed to determine at what $[\text{Ca}_i^{++}]$ the PMN are able to be stimulated with Con A to produce O_2^- . PMN were depleted of Ca^{++} by loading with Quin2 as described above. The PMN were then incubated with Con A for two minutes at 37 $^\circ\text{C}$ in the presence of cytochalasin B. At that time 1 mmol/L Ca^{++} was added, and the lag time

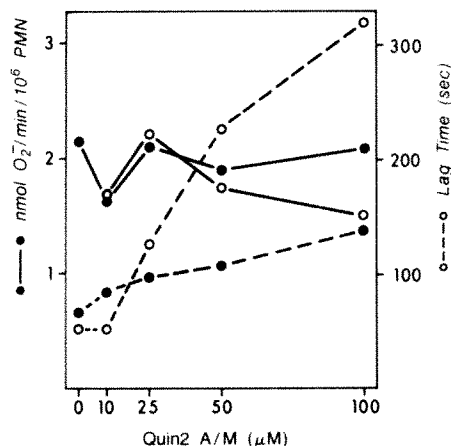


Fig 7. Rate of O_2^- production and lag time by Con A-treated Ca^{++} -depleted PMN in the presence of 10 $\mu\text{mol/L}$ cytochalasin B. PMN were depleted of Ca^{++} by incubation with the indicated concentrations of Quin2 A/M as described in "Methods." O_2^- production was measured in HBS as indicated. Closed symbols indicate PMN that had been repleted with 1 mmol/L CaCl_2 for ten minutes at 37 $^\circ\text{C}$ prior to the addition of Con A. Open symbols indicate PMN that were treated with Con A for two minutes prior to the addition of 1 mmol/L CaCl_2 . Solid lines indicate the rate of O_2^- production. Dashed lines indicate the lag time prior to O_2^- production, which for the solid symbols is calculated from the time of addition of Con A, and for the open symbols is calculated from the time of addition of Ca^{++} .

prior to O_2^- production was measured. As can be seen in Fig 8, when the lag times are compared with the time tracing for the repletion of $[\text{Ca}_i^{++}]$ to these same Ca^{++} -depleted PMN, the lag time for O_2^- production always corresponded to a $[\text{Ca}_i^{++}]$ of approximately 79 nmol/L . This would indicate that activation of the O_2^- generating system by Con A can occur at $[\text{Ca}_i^{++}]$ that is lower than has generally been appreciated.

DISCUSSION

Several laboratories have recently been reporting a phenomenon in PMN termed "priming." In general, priming involves the sequential use of different stimuli, each added at suboptimal concentrations, resulting in a synergistic activation of PMN. For example, PMN treated with low concentrations of PMA followed by FMLP generate O_2^- at a rate far greater than either stimulus alone.^{30,31} This same type of phenomenon has also been reported using PMA and the Ca^{++} ionophore ionomycin.²³

Con A is useful in the study of PMN priming because of the ability of $\alpha\text{-MM}$ to inhibit the binding of Con A to the cell surface. O_2^- production by Con A-treated PMN can therefore be prematurely terminated by the addition of $\alpha\text{-MM}$.¹³⁻¹⁵ While O_2^- production is terminated by this procedure and can be reactivated by an additional stimulus, some other responses are not reversed. The Con A-induced membrane potential depolarization is not reversed and degranulation continues.¹⁵ These cells do not return to a true resting state and appear to be "primed" for reactivation because lag times prior to O_2^- production are shorter in previously activated PMN than for control PMN. In addition, these previously activated PMN have a diminished requirement for extracellular calcium when reactivated with Con A.¹⁵ This led us to speculate that permanent changes in $[\text{Ca}_i^{++}]$ might account for the priming in this system.

Recent reports have utilized the intracellular Ca^{++} indicator Quin2 to monitor changes in phagocyte $[\text{Ca}_i^{++}]$. Using this technique, rapid stimulus-induced increases in $[\text{Ca}_i^{++}]$ are reported for PMN stimulated with chemotactic pep-

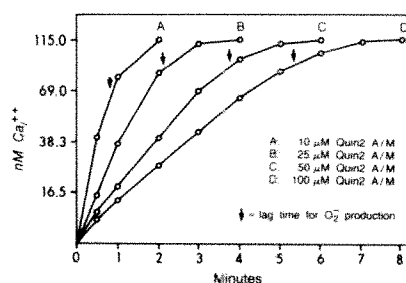


Fig 8. Relationship between lag times for O_2^- production with $[\text{Ca}_i^{++}]$ in Ca^{++} -depleted, Con A-treated PMN. PMN was depleted of Ca^{++} as described in "Methods." Fluorescence tracings and O_2^- production lag times were determined in HBS. In all samples 100 $\mu\text{g/mL}$ Con A was added to PMN in the presence of 10 $\mu\text{mol/L}$ cytochalasin B for two minutes followed by the addition of 1 mmol/L CaCl_2 . The arrows for each curve indicate the lag time for O_2^- production for that sample. Shown is a representative experiment. For three experiments, the $[\text{Ca}_i^{++}]$ at which O_2^- production induced by Con A is $79 \pm 20 \text{ nmol/L}$ (mean $\pm$ SD).

tides,^{11,12,24} monocytes²⁹ and PMN^{12,25} stimulated with Con A, and J774 macrophages stimulated with ligands for immunoglobulin Fc receptors.²² An increase in $[Ca_i^{++}]$ is not sufficient to induce O_2^- production by PMN, since elevation of $[Ca_i^{++}]$ with the Ca^{++} ionophore ionomycin does not induce O_2^- production¹¹ and because chemotactic concentrations of FMLP induce a maximal increase in $[Ca_i^{++}]$ but do not induce O_2^- production.^{12,32}

Our data show that the activation of PMN by Con A results in a sustained increase in $[Ca_i^{++}]$ similar to that seen in monocytes²⁹ and in contrast to the transient increase in PMN $[Ca_i^{++}]$ induced by FMLP.^{11,12,24} This sustained increase in $[Ca_i^{++}]$ can be reversed when α -MM is added to Con A-treated PMN. PMN treated in this manner return to the original resting $[Ca_i^{++}]$. These activated and inactivated PMN can then be reactivated by the addition of a second stimulus such as arachidonic acid, resulting in a second increase in $[Ca_i^{++}]$. This result is important because the priming effect seen in PMN treated with Con A followed by α -MM cannot therefore be due to permanent elevations in $[Ca_i^{++}]$. However, these results do confirm that $[Ca_i^{++}]$ is tightly coupled to the activation, inactivation, and reactivation of PMN and that increments in $[Ca_i^{++}]$ occur every time PMN are activated.

We performed the same type of experiment using granule-depleted and enucleated PMN cytoplasts. Cytoplasts activated with Con A to produce O_2^- can be inactivated with α -MM and reactivated with the addition of another stimulus.²⁸ As measured using Quin2, $[Ca_i^{++}]$ of these cytoplasts displayed only a very small increase over several minutes after treatment with Con A. In one previous report, cytoplasts treated with complement fragment C5a did not generate O_2^- or display an increase in $[Ca_i^{++}]$,²⁴ while in another report cytoplasts treated with FMLP did generate O_2^- but displayed only a very small increase in $[Ca_i^{++}]$.³³ The small change in $[Ca_i^{++}]$ in Con A-treated cytoplasts was inhibitable and reversible by α -MM, but the result raised questions about what level of $[Ca_i^{++}]$ is needed for PMN activation by Con A. Although results using chlortetracycline to monitor changes in membrane-associated calcium are difficult to quantitate,^{9,10} it is noteworthy that the time course of fluorescence changes in chlortetracycline-loaded PMN and cytoplasts treated with Con A were similar, and the time course of fluorescence changes of Quin2-loaded cytoplasts in response to Con A were much slower than the changes in control PMN. This raises the possibility that changes in membrane-bound calcium in Con A-treated cytoplasts occur in a time frame consistent with a role in the activation of the cytoplasmic O_2^- generating system. It is also possible that an increment in $[Ca_i^{++}]$ and not an absolute level of $[Ca_i^{++}]$ is the important event in the activation of PMN by Con A.

PMN could be reversibly depleted by $[Ca_i^{++}]$ by loading with Quin2 in the absence of extracellular Ca^{++} . These Ca^{++} -depleted PMN did not generate O_2^- or undergo an increase in $[Ca_i^{++}]$ after treatment with Con A in the absence of extracellular Ca^{++} . The addition of Ca^{++} to these PMN resulted in the return of $[Ca_i^{++}]$ to the original resting level. The rate of the return of $[Ca_i^{++}]$ to resting levels was influenced by the Ca^{++} -buffering action of intracellular

Quin2. This allows the determination of the $[Ca_i^{++}]$ at which O_2^- production begins in response to Con A. Over a tenfold range of intracellular Quin2 concentration, O_2^- production always began at the same $[Ca_i^{++}]$ (approximately 79 nmol/L Ca_i^{++}), which is lower than the resting $[Ca_i^{++}]$ of normal Ca^{++} -replete PMN. This raises the question about whether $[Ca_i^{++}]$ levels above the normal resting $[Ca_i^{++}]$ are needed for activation of PMN by Con A.

Sklar and Oades have also recently reported that Ca^{++} -depleted PMN can generate O_2^- in response to chemotactic peptides at $[Ca_i^{++}]$ that are lower than the normal resting $[Ca_i^{++}]$.³⁴ However, in contrast to our results, they found that increasing rates of O_2^- were obtained with Ca^{++} -depleted PMN that had been repleted to increasing levels of $[Ca_i^{++}]$. Our experiments were possible because Ca^{++} -depleted PMN were absolutely unable to generate O_2^- unless $[Ca_i^{++}]$ had been allowed to increase to a threshold level. Since O_2^- production began at the same indicated $[Ca_i^{++}]$ over a tenfold range of intracellular Quin2, it is likely that all of the PMN were being activated. It is possible that in the studies of Sklar and Oades, using chemotactic peptides, only a fraction of the PMN were generating O_2^- and that these PMN had $[Ca_i^{++}]$ that were higher than the average value obtained in this type of measurement. Another possibility is that the Ca^{++} -dependent transduction mechanisms for Con A and chemotactic peptide stimulation are different, allowing O_2^- to proceed at different $[Ca_i^{++}]$. Supporting this possibility of divergent transduction mechanisms is the report that demonstrates different sensitivity of Con A and chemotactic peptide stimulation to pretreatment of PMN with pertussis toxin.³⁵ These questions will be best answered using the recently developed fluorescent Ca^{++} indicators that are suitable for use in laser-activated flow cytometry.³⁶

It has been reported that PMA-induced activation of PMN protein kinase C can occur at approximately 10 nmol/L $[Ca_i^{++}]$.²³ Thus it appears that some PMN responses can occur at $[Ca_i^{++}]$ that are lower than resting levels, even though increments in $[Ca_i^{++}]$ have been shown to correlate with activation of PMN by all stimuli with the exception of PMA.³⁷ In contrast, it has been reported that granule secretion by PMN that have been permeabilized with either saponin or digitonin is induced by micromolar levels of Ca^{++} .^{38,39} Levels of $[Ca_i^{++}]$ of this magnitude are not required for the activation of Con A-induced O_2^- production by either PMN or cytoplasts. It is still unclear whether stimulus-induced increases in $[Ca_i^{++}]$ are activating events (or promoters of activation) of O_2^- production. It may be that changes in membrane-associated calcium are activating events in PMN and result in an increase in $[Ca_i^{++}]$. Only identification of the PMN components that are either activated or inactivated by calcium will clarify this issue.

ACKNOWLEDGMENT

We are indebted to Drs Sean Scully and George Segel for discussions on the role of intracellular free calcium in phagocytes.

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Inactivation of Single-Chain Urokinase (Pro-urokinase) by Thrombin and Thrombin-like Enzymes: Relevance of the Findings to the Interpretation of Fibrin-Binding Experiments

By Victor Gurewich and Ralph Pannell

Whereas crude bovine thrombin activated single-chain urokinase-type plasminogen activator (scu-PA), otherwise called pro-urokinase (pro-UK), purified human thrombin converted pro-UK (scu-PA) to a two-chain form that had no amidolytic activity. The two chains ($M_r \sim 33,000$ and 22,000) were disulfide linked and resistant to subsequent activation by plasmin. By contrast, thrombin did not inactivate tissue plasminogen activator or two-chain urokinase. The enzyme from snake venom *Agkistrodon contortrix*, relatively specific for fibrinopeptide B, had an effect similar to thrombin, whereas the enzyme from *Agkistrodon rhodostoma* (ancrod), specific for fibrinopeptide A, did not. When pro-UK (scu-PA) was present during thrombin clotting of fibrinogen, degradation of ^{125}I -pro-UK (scu-PA) in the clot supernatant was seen, whereas virtually full recovery (95%) of radioactivity was found. A loss of latent

amidolytic activity in the clot supernatant was also found, the extent of which could be correlated with the degree of degradation of the radiolabeled probe. It was concluded that thrombin inactivation of pro-UK (scu-PA) accounts for the loss of amidolytic activity in the clot supernatant, which has been attributed to fibrin binding. Further confirmation was obtained from experiments in which ancrod was used as the clotting agent. Full recovery of both radioactivity and latent amidolytic activity of pro-UK (scu-PA) in the supernatant was obtained under these conditions. These findings indicate that thrombin may introduce an artifact in the results of certain experiments designed to study the fibrin affinity or fibrinolytic effect of pro-UK (scu-PA).

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WE HAVE PREVIOUSLY REPORTED that, in contrast to the commonly used crude bovine thrombin preparation, Thrombostat (Parke Davis, Avon, CT), purified human thrombin did not activate single-chain urokinase-type plasminogen activator (scu-PA), otherwise called pro-urokinase (pro-UK).¹ A contaminant in the crude bovine thrombin was considered responsible for its effect on pro-UK (scu-PA).

Recently Conforti et al reported that treatment of pro-UK (scu-PA) with purified thrombin inhibited the plasmin activatability of pro-UK (scu-PA).² On more systematic analysis we have found that purified human thrombin and one thrombin-like enzyme from snake venom degrade and inactivate pro-UK (scu-PA) but not tissue plasminogen activator (t-PA) or two-chain urokinase (TC-UK). The relevance of these findings to the interpretation of certain pro-UK/fibrin binding and clot lysis experiments is presented.

EXPERIMENTAL PROCEDURES

Materials. Pro-urokinase (99% single chain), purified from the culture medium of a transformed human kidney cell line was obtained from Collaborative Research, Lexington, MA. High mol wt TC-UK, originating from Green Cross (Osaka, Japan), was obtained as a gift from Dr Bleakley Chandler. Tissue plasminogen activator, predominantly single chain, was obtained from Integrated Genetics (Framingham, MA). Fibrinogen and amidolytic substrate (S-2444) were obtained from Kabi Vitrum (Stockholm, Sweden). Highly purified human thrombin (1623 NIH U/mg) was a gift of Dr John Fenton to Dr Robert Weinstein of this department. Ancrod (from *Agkistrodon rhodostoma* venom) was obtained from Sigma (St. Louis). The thrombin inhibitor N- α -(2-naphthylsulfonyl)-glycyl-D,L-amidinophenylalaninepiperidine (NAPAP) and the thrombin-like enzyme Contortrix, from the venom of *Agkistrodon contortrix*, were obtained from American Diagnostica (Greenwich, CT). Phenylalanyl-prolyl-arginyl-chloromethylketone (PPack) and bovine serum albumin, "Purified" grade, were obtained from Calbiochem (San Diego, CA).

Plasminogen-free fibrinogen was prepared from Kabi fibrinogen by DFP (5 mmol/L) treatment followed by chromatography on Lysine-Sepharose³ equilibrated with 20 mmol/L trisodium citrate, 0.15 mol/L NaCl, and, finally, dialysis against 25 mmol/L HEPES,

pH 7.4, 0.15 mol/L NaCl. The preparation was >99% clottable protein.

Radiolabeling. Pro-UK (scu-PA) and TC-UK were labeled with ^{125}I via the lactoperoxidase reaction using immobilized lactoperoxidase/glucose oxidase (Enzymobeads, BioRad Laboratories) as previously described.⁴ The resulting specific activities were approximately 30 $\mu\text{Ci}/\mu\text{g}$. Labeling was accompanied by some loss of enzymatic activity, but the labeled pro-UK could be fully cleaved by plasmin at a rate comparable to unlabeled pro-UK (scu-PA).

Amidolytic assay of pro-UK (scu-PA) or TC-UK. Amidolytic activity was measured with Kabi substrate S-2444 (0.75 mmol/L) at 37 °C. The reaction buffer was 0.1 mol/L Tris-HCl (pH 8.8), 0.1 mol/L NaCl and 0.1 mg/mL BSA. One enzyme unit (EU) was defined as the amount of enzyme producing a change in absorbance (405 nm) of 1 absorbance unit per minute. Activation of pro-UK (scu-PA) (0.45 $\mu\text{mol/L}$) to TC-UK was accomplished by incubation (37 °C) with plasmin (0.125 $\mu\text{mol/L}$) for 30 to 45 minutes. The amidolytic activity (latent activity) in the mixture was measured with the addition of aprotinin (200 KIU/mL) to the reaction buffer.

Treatment with thrombin or thrombin-like enzymes. The latent amidolytic activity of pro-UK (scu-PA) was measured at intervals after incubation (37 °C) at a concentration of 5,000 IU/mL (50 $\mu\text{g/mL}$) with thrombin (0.01 to 10 NIH U/mL). Similar experiments were carried out with TC-UK (5,000 IU/mL) and with t-PA (50 $\mu\text{g/mL}$).

The effect of the thrombin-like enzymes from *Agkistrodon*

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venoms on the amidolytic activity of pro-UK (scu-PA) were also examined in a similar manner: ancred (specific for fibrinopeptide A),⁵ 5 U/mL; and Contortrix (relatively specific for fibrinopeptide B),⁶ 200 mU/mL (chromogenic assay units as defined by the supplier). These concentrations gave clotting times of five to ten minutes under the conditions used in the fibrin clot-binding experiments.

The effect of thrombin inhibitors on the degradation of pro-UK (scu-PA) by thrombin was tested. Thrombin (10 NIH U/mL) was pretreated with PPAck (20 μ mol/L) for one hour at 37 °C. The competitive inhibitor NAPAP was present at 10 μ mol/L during the thrombin treatment of pro-UK (scu-PA). Aprotinin was tested at 500 KIU/mL present during the thrombin treatment.

The fate of pro-UK (scu-PA) during fibrin clot-binding experiments. Fibrin-clot-binding experiments were performed by an extension of the basic method described by Kasai et al.⁷ In brief, pro-UK (scu-PA) or TC-UK (30 to 45 IU/mL) were added to a reaction mixture of plasminogen-free fibrinogen (2 mg/mL), 0.15 mol/L NaCl, 25 mmol/L HEPES (pH 7.4), 0.01% Tween 80, and 0.2% bovine serum albumin. The mixture was clotted with thrombin (1 NIH U/mL, final concentration) or ancred (5 U/mL) and incubated (37 °C) for 15 minutes. The tubes were chilled on ice, vortexed, and centrifuged. The plasminogen activator activity was measured in the supernatant and compared with the activity in tubes without fibrinogen and tubes without thrombin. The average results from five experiments were calculated.

Additional experiments were performed with radiolabeled pro-UK (scu-PA) or TC-UK added to the above mixture. The supernatant and control tubes were analyzed by autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as for radioactivity.

SDS-PAGE. Samples from the above reaction mixtures were subjected to SDS-PAGE by the method of Laemmli⁸ using 10% gels. Samples were prepared with or without 10 mmol/L dithiothreitol and stained with Coomassie brilliant blue G-250 or visualized by autoradiography.

RESULTS

Thrombin inactivation of the latent activity of pro-UK (scu-PA). When pro-UK (scu-PA) was incubated with purified human thrombin, there was a time and thrombin concentration-dependent loss of the latent UK amidolytic activity (generated by subsequent plasmin treatment of the solution) as shown (Fig 1). Pro-UK (scu-PA) was completely protected by NAPAP when it was included in the incubation

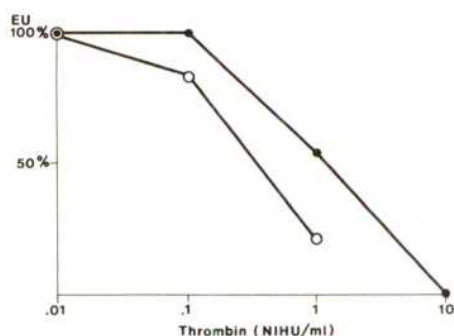


Fig 1. Latent amidolytic activity of pro-UK (scu-PA), expressed as percent of baseline enzyme units (EU), recovered after incubation (37 °C) with purified human thrombin (0.01 to 10 NIH U/mL) for 15 (●) and for 60 minutes (○).

mixture; there was only 28% loss of activity when PPAck was used; and aprotinin had no effect on the reaction.

The amidolytic activities of TC-UK and of t-PA were unaffected by incubation with thrombin (10 NIH U/mL for 30 minutes).

Effect of ancred or contortrix venom on the latent amidolytic activity of pro-UK (scu-PA). The latent amidolytic activity of pro-UK (scu-PA) was not affected by incubation (37 °C) with ancred (5 U/mL) for 30 minutes. By contrast, Contortrix venom (200 MU/mL) induced a progressive loss of the latent amidolytic activity of pro-UK (scu-PA), which was comparable to that induced by thrombin. The venom had no effect on the amidolytic activities of TC-UK or t-PA.

Thrombin degradation of pro-UK (scu-PA). A mixture of pro-UK (scu-PA) (5,000 IU or 50 μ g/mL) and thrombin (1 NIH U/mL) sampled at 10, 20, 30, 45 and 60 minutes for SDS-PAGE under reducing conditions, showed progressive degradation of single chain pro-UK (scu-PA) to two chains corresponding to about M_r 33,000 and 22,000. The two chains appeared to migrate just slightly behind the heavy and light chains of the TC-UK marker. A relatively faint higher molecular weight contaminant is also seen (Fig 2).

When the appearance of thrombin-modified pro-UK (scu-PA) under reducing and nonreducing conditions was compared (Fig 3), the thrombin-modified pro-UK (scu-PA) migrated with a M_r of 55,000 under nonreducing conditions (lane 2), indicating that the thrombin cleavage peptides were disulfide linked. The human thrombin appeared as a major and minor band (lane 3) and the pro-UK (scu-PA) as a single band under reducing conditions (lane 1). The higher mol wt contaminant seen in Figure 2 is again seen in the pro-UK-thrombin mixture under reducing conditions (lane 2, Fig 3).

Inactivation and degradation of pro-UK (scu-PA) during fibrin clotting. In the tubes containing buffer, thrombin, and pro-UK (scu-PA), a 49% (mean) loss from control tubes (without thrombin) of the latent amidolytic activity of pro-UK (scu-PA) was found, whereas a 27% (mean) loss was

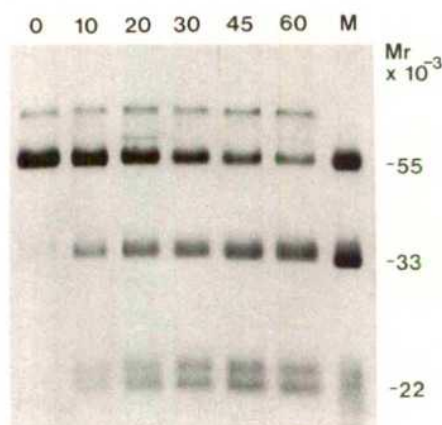


Fig 2. Reduced SDS-PAGE of M_r 55,000 pro-UK (scu-PA) (50 μ g) incubated (37 °C) with thrombin (1 NIH U/mL) for 0, 10, 20, 30, 45, and 60 minutes. A marker (M) made up of a mixture of pro-UK (scu-PA) and TC-UK is shown. The high mol wt band is a contaminant having no time dependence.

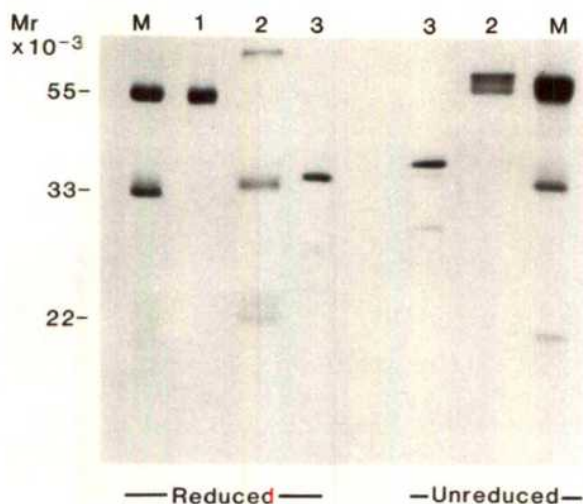


Fig 3. SDS-PAGE under reducing and nonreducing conditions of pro-UK (scu-PA) before (1) and after (2) incubation (37 °C) with thrombin (5 NIH U/mL) for 60 minutes. The human thrombin preparation (5 µg) (3) and a marker (M) made up of a mixture of pro-UK (scu-PA) and TC-UK are shown. A high mol wt band, comparable to the contaminant seen in Fig 2, can be seen in lane 2 (reduced) but not elsewhere.

found in the fibrin clot supernatant. No significant change in the amidolytic activity of TC-UK was observed either in buffer or in the fibrin clot supernatant. A 95% recovery of radioactivity from ^{125}I -pro-UK (scu-PA) and complete recovery of ^{125}I -TC-UK in the clot supernatant were obtained (Table 1).

Autoradiography of SDS-PAGE of incubation mixtures from this experiment showed degradation of ^{125}I -pro-UK (scu-PA) during incubation with thrombin both with and without fibrinogen. Loss of the M_r 55,000-band compared with baseline and appearance of a M_r 33,000-band was seen (Fig 4). The degradation was greater in buffer (lanes 2 and 3) than in the fibrin clot supernatant (lanes 5 and 6). Samples from two experiments with pro-UK are shown to demonstrate the reproducibility of the findings (lanes 2, 3, 5, and 6). There was no change in the appearance of TC-UK (lanes 1 and 4) (Fig 4).

When anacrod (5 U/mL) was used instead of thrombin, there was no loss of either amidolytic activity or radioactivity in the fibrin clot supernatant.

Table 1. Amidolytic Activity and Radioactivity After 15 Minutes Incubation (37 °C) with Thrombin (1 NIH U/mL) or after Thrombin Clotting

	Pro-UK Amidolytic Action Radioactivity		TC-UK Amidolytic Action Radioactivity	
	EU/mL	CPM	EU/mL	CPM
Control	0.98 (100%)	4,175 (100%)	0.110 (100%)	4,015 (100%)
Thrombin + Buffer	0.050 (51%)	4,175 (100%)	0.104 (95%)	4,015 (100%)
Clot Supernatant	0.072 (73%)	3,986 (95%)	0.107 (97%)	4,084 (102%)

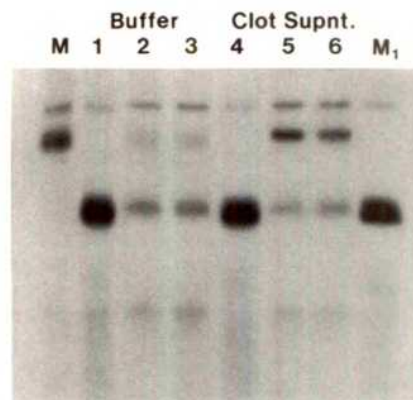


Fig 4. Autoradiograph of reduced SDS-PAGE of ^{125}I -pro-UK (scu-PA) (M) after 15 minutes incubation (37 °C) with thrombin (1 NIH U/mL) in buffer (2 and 3) or in fibrinogen solution (clot supernatant) (5 and 6). Samples from two separate clots are shown. The results of similar mixtures with ^{125}I -TC-UK (M1) are seen in lanes 1 and 4 representing buffer and clot supernatant respectively.

DISCUSSION

This study demonstrates that the latent activity of pro-UK (scu-PA) but not the activities of TC-UK or of single chain t-PA, is inactivated progressively by physiologic concentrations of thrombin. Thrombin converted pro-UK (scu-PA) to a two-chain form that had no amidolytic activity.

The two chains generated by thrombin migrated on SDS-PAGE in positions comparable to those of the light and heavy chains of TC-UK. This molecule was not susceptible to activation by plasmin. Conversely, TC-UK was not inactivated by thrombin. The thrombin cleavage site in pro-UK (scu-PA) was close to the plasmin cleavage site within the disulfide-linked domain of the molecule. Inactivation of pro-UK (scu-PA), comparable to that induced by thrombin, was also seen with the thrombin-like snake venom enzyme from Agkistrodon contortrix, relatively specific for fibrinopeptide B, but not with the one from Agkistrodon rhodostoma (anacrod), specific for fibrinopeptide A.

When pro-UK (scu-PA) was incubated with thrombin plus fibrinogen, there was a loss of latent activity in the clot supernatant relative to a buffer control without thrombin. But this loss of activity in the supernatant, previously attributed to fibrin binding,⁸ could be correlated with the degree of degradation seen by SDS-PAGE autoradiography. Further evidence that the pro-UK (scu-PA) did not bind to fibrin under these conditions was the full recovery of both counts from ^{125}I -pro-UK (scu-PA) and plasmin-activatable amidolytic activity in the supernatant when anacrod was used instead of thrombin to clot fibrinogen. Thus in this experimental model, thrombin inactivation of pro-UK (scu-PA) introduces an artifact that may masquerade as fibrin binding.

Conversely, when fibrin affinity is determined by the recovery of UK-antigen after clotting,⁹ degradation of pro-UK (scu-PA) would obscure fibrin binding under experimental conditions in which the fibrin binding of pro-UK itself may occur. The sensitivity of pro-UK (scu-PA) to degrada-

tion by thrombin may also lead to an underestimation of its contribution to lysis when this is studied by measuring spontaneous fibrinolysis after thrombin clotting.¹⁰

The addition of fibrinogen attenuated the inactivation and degradation of pro-UK (scu-PA) by thrombin. This was observed in the fibrin clot-binding experimental model in which the extent of degradation found in the clot supernatant was about 45% less than that in the buffer control. This effect may be explained by competition of the substrate fibrinogen (6 $\mu\text{mol/L}$) with the substrate pro-UK (scu-PA) (9 nmol/L) for the active site of thrombin.

During the finalization of this manuscript, the cleavage of pro-UK (scu-PA) by thrombin at the Arg 156-Phe 157 bond, two residues from the activation site, was identified by Ichinose et al.¹¹ In view of this, it is noteworthy that cleavage at this site renders the Lys 158-Ile 159 bond not susceptible to activation by plasmin. Perhaps not surprisingly, cleavage within the disulfide-linked domain of pro-UK causes a major conformational change in this region, causing the susceptible bonds for plasmin and respectively for thrombin to become nonrecognizable.

This effect of thrombin explains an earlier observation that pro-UK (scu-PA), in contrast to UK or streptokinase, induces little endolysis when incorporated into a forming clot (unpublished), whereas pro-UK (scu-PA) induces exolysis of a preformed clot incubated in plasma very effectively.¹ Physiologically the inactivation of pro-UK (scu-PA) by

thrombin may serve to maintain the integrity of a fresh clot by inhibiting endolysis. Tissue plasminogen activator and TC-UK, though resistant to thrombin, are inhibited by plasminogen activator inhibitor-1,¹² whereas pro-UK is not.¹³ Therefore a separate mechanism appears to exist in a forming clot to inactivate pro-UK (scu-PA) and to prevent the generation of TC-UK.

These findings are relevant to the interpretation of certain published studies but do not resolve the controversy regarding the fibrin binding of pro-UK (scu-PA). They only suggest that pro-UK (scu-PA) does not bind to fibrin under the present experimental conditions. Since a single chain form of UK was first identified in urine and isolated because of its specific binding to fibrin/Celite,¹⁴⁻¹⁶ a certain fibrin affinity for pro-UK (scu-PA) has been assumed. However, this property of pro-UK appears to be conditional, as suggested by the observation of Husain¹⁷ that Zn^{++} is a requirement. We have recently confirmed this Zn^{++} dependence using fibrin-lined tubes pretreated with an antithrombin. Binding of pro-UK (scu-PA) to fibrin/Celite was also confirmed in this study and was shown to be potentiated by Zn^{++} , in contrast to TC-UK, which did not bind and was unaffected by Zn^{++} .¹⁸

In conclusion, the sensitivity of pro-UK (scu-PA) to degradation by thrombin is relevant to the interpretation of some experimental findings and probably is involved in modulating the biologic function of pro-UK (scu-PA).

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Large Numbers of Primitive Stem Cells Are Active Simultaneously in Aggregated Embryo Chimeric Mice

By D.E. Harrison, C. Lerner, P.C. Hoppe, G.A. Carlson, and D. Alling

The possibility has been repeatedly raised that erythropoiesis results from clonal succession—the differentiation of one or a very small number of the most primitive stem cells that are sequentially activated to proliferate forming clones of differentiated cells and then eventually decline, to be replaced by new stem cell clones. We studied this possibility in chimeric mice made by combining embryos from two different strains so that they would have two distinct stem cell populations, each of which produces a different hemoglobin type (d and s). These were compared with F_1 hybrids in which every stem cell produces both types. We measured the percentage of type d in seven to ten serial samples of circulating reticulocytes taken at three- to seven-day intervals and found that the variability in percent of this hemoglobin was only slightly higher in the chimeric mice than in F_1 controls; SD ranged from 2.7% to 5.5% in the chimeric mice and from 3.4% to 3.9% in the controls. Using the binomial formula, the numbers of new clones formed during the reticulocyte life span, approximately three days, ranged from 33 to 118 in the individual

chimeric mice. However, these numbers are underestimates because estimated numbers of clones depend inversely on variabilities, and the calculations did not exclude the contribution of experimental error to the overall variability. Total percentages of type d hemoglobin were also measured in seven to nine successive serial samples at 60- to 136-day intervals. These gave mean values similar to measures of newly synthesized hemoglobin in the same mice, but SD were larger, ranging from 5.3% to 8.4%. This reflects experimental error, both because of excess day-to-day variability found in this type of measurement and because there could not be fewer primitive stem cells activated to form clones of erythrocytes during the 45-day erythrocyte life span than during the three-day life span of reticulocytes. Since most and maybe all of the variation between successive samples in the same chimeric mouse appear to result from experimental error, many or even all of the primitive stem cells may simultaneously contribute to erythropoiesis.

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THE HYPOTHESIS that normally functioning somatic cells have a limited proliferative capacity¹ appears to be contradicted by findings that long-term repopulating abilities of stem cells are unchanged after a life span of normal function.²⁻⁴ However, only a small fraction of differentiating cells are primitive stem cells, so the kinetic patterns by which they multiply and differentiate have not been defined unambiguously. Important basic information such as the number of stem cells in a marrow graft or the number of doublings that can be achieved by hematopoietic cells cannot be estimated without knowing these patterns. It is possible that most stem cells proliferate very little, so that at any given time only a few contribute to erythropoiesis.⁵⁻¹⁰ This would allow the remaining stem cells to conserve their proliferative capacity.

Numbers of clones contributing to a population made up of two cell types may be estimated using the binomial formula^{8,11} in which the estimated number of clones (n) depends inversely on the variance of the observations (see below). Numbers calculated in this way are underestimates because the variance includes experimental error and is thereby increased so that n is decreased. The estimated number of stem cells is also decreased if any other source of variability within the individual mice is increased. Use of this calculation in our studies is based on the following assumptions: that the different phenotypes in reticulocytes and in mature erythrocytes will occur in the same proportions as those of the active primitive stem cells; that no other mechanisms influence the proportioning of cells of the different genotypes; and that each primitive stem cell becomes inactive after it produces its burst of erythrocytes.

To obtain cells with two distinguishable hemoglobin types, we made chimeric mice by aggregation of early embryos of two distinguishable genotypes. Such mice are normal appearing and may contain cells of both genotypes in any tissue, including the hematopoietic system.¹²⁻¹⁵ Relative pro-

portions of blood cells in such mice may favor one genotype, a tendency that is reduced by using congenic strains.¹⁶⁻¹⁸ Changes with time in populations of RBCs and WBCs and cells in lymphoid tissues within a single mouse have been found in 20% to 60% of chimeras.^{14,19-21} In these "unstable" chimeras, peripheral WBCs and hemoglobin compositions were not correlated.¹⁹ In one set of studies there was no apparent preference for cells of one of the genotypes composing the chimera,²¹ while in a previous report there was a shift to the B6 genotype.¹⁴

Previously reported theories and data implied that very few stem cells produce erythroid cell clones at the same time, and each is active for a short time.⁵⁻¹⁰ If this is true, then much more variability should be observed when newly synthesized rather than total hemoglobin proportions are monitored, since RBCs newly released into the circulation only synthesize hemoglobin for three days and hence are much less subject to the damping effect observed in mature erythrocytes that have overlapping 45-day life spans. The genotypes of both newly released and total RBCs are controlled

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by the genotype of the most primitive stem cells from which the blood cells are descended. Therefore we followed changes with time in populations of hemoglobin-synthesizing reticulocytes as well as total RBCs in mice containing cells that produce two distinguishable hemoglobin types.

MATERIALS AND METHODS

Chimeric mice were made from the C57BL/6J (B6) and CBA/HT6J (CBA) inbred strains. B6 and CBA embryos were aggregated at an early preimplantation stage, and the aggregate was allowed to develop to term in a foster mother, using techniques previously described.¹⁵ The B6 and CBA strains have hemoglobin types that are distinguishable by electrophoresis; at the beta chain (Hbb) locus, B6 mice have *Hbb^s* (s, single) hemoglobin, which forms the band migrating farthest on the cellulose acetate gels used, whereas CBA mice have *Hbb-b1^d* and *Hbb-b2^d* (d, diffuse) hemoglobins. These are the intermediate—and slowest—migrating bands, respectively. Only seven of the 24 chimeras consistently had detectable levels of type d bands and were used for these experiments. Coat colors of six of these were roughly equal mixtures of CBA type (agouti) and B6 type (black), while one had only agouti hair. Seven other chimeras with roughly equal mixtures of CBA and B6 coat types produced only B6-type hemoglobin, as did all ten mice with only B6-type coats. There may have been no CBA cells in the latter. All mice were produced and maintained at the Jackson Laboratory, which is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Reticulocytes in circulating blood cells from these mice were incubated with ³⁵S-labeled methionine to quantitate their hemoglobin synthesis as follows. Peripheral blood was collected from the retro-orbital sinuses, removing 30 μ L in 10 μ L and 20 μ L capillary tubes. The 10 μ L sample was used to determine hemoglobin concentrations to assure that mice did not become anemic with repeated sampling. Hemoglobin synthesizing activity also did not increase with repeated sampling, indicating that reticulocyte numbers did not significantly increase, since only small amounts of blood were used. The 20 μ L sample was added to 100 μ L of F-12M medium (#12-615A, MA Bioproducts, Walkersville, MD) containing 2% fetal calf serum and 50 μ Ci of [³⁵S]-labeled methionine (#SJ-204, Amersham, Arlington Heights, IL) to label freshly synthesized hemoglobin as previously described.^{4,22} Cultures were incubated for three hours at 37 °C in 5% carbon dioxide, balance air.

Hemoglobins were separated by standard electrophoretic procedures as follows: The blood cells were washed twice with cold saline by centrifugation, and the pellet was resuspended in 20 μ L of the Cystamine lysing agent used to prepare hemoglobins for electrophoresis. Samples were frozen to lyse the cells, and the hemoglobin types were separated by electrophoresis on cellulose acetate gels as previously described.^{4,23} These d and s bands are illustrated in Fig 1.

Total amounts of radioactive d and s hemoglobins were determined, and the relative amount of labeled d hemoglobin was expressed as a percent as follows. Each of the hemoglobin-containing bands was cut from the cleared gel, solubilized in 1 mL of Soluene-350 (Packard, #6003038 diluted 9 to 1 with distilled water), and the [³⁵S] activity was counted by scintillation counting with an Omniflor cocktail (#NEF-906B, New England Nuclear, Boston). Background was determined by counting a portion of the gel beyond where the hemoglobin migrated, and background counts were subtracted. Data were expressed as the percentage (counts in diffuse bands/total counts in the diffuse + single bands). Identical separation procedures were used for measurements of total hemoglobins, but packed RBCs were used, and amounts of d and s hemoglobin were determined by densitometry, as previously described.^{4,23}

Two-color analysis by flow cytometry was used to determine

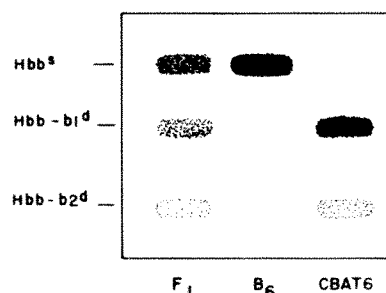


Fig 1. The bands pictured are *Hbb-b1^d* (was *Hbb^{dms}*) and *Hbb-b2^d* (was *Hbb^{dms}*), containing 80% and 20% respectively of the d murine hemoglobin and the s band of s (single or *Hbbs*) hemoglobin. This figure diagrams a typical cellulose acetate electrophoretic gel.

the percentage of *H-2^b/H-2^b* lymphocytes in peripheral blood. Briefly, lymphocytes were isolated from 150 μ L of peripheral blood by centrifugation through Ficoll-Hypaque (LSM, Bionetics Laboratory Products, Kensington, MD) and incubated in 200 μ L with labeled anti-H-2D^b (28-14-8) and anti-H-2K^k (16-3-1) monoclonal antibodies (MoAbs).²⁴ Reciprocal determinations were made using fluoresceinated anti-H-2K^k with biotinylated anti-H-2D^b and biotinylated anti-H-2K^k with fluoresceinated anti-H-2D^b to ensure the accuracy of the results. After washing, binding of the biotinylated antibody was revealed by incubation with phycoerythrin-conjugated avidin (Becton Dickinson, Mountain View, CA). The cells were then analyzed using an Ortho Instruments Model 50H Cytofluorograph with a Model 2140 Data Handling System. Controls included CBA/HT5 and C57BL/6J lymphocytes and a series of different mixtures of the two. Overlap between red and green fluorescence was less than 1%.

RESULTS

Table 1 shows the percentages of ³⁵S-methionine incorporated into hemoglobins newly synthesized by reticulocytes. Seven to ten blood samples were serially tested from each of

Table 1. Newly Synthesized CBA-Type (d-Type) Hemoglobin Percentages in Seven Chimeric and Four F₁ Hybrid Mice

Mouse Type	Identification No.	Percent labeled CBA-type HB*	Calculated No. Clones
Chimeric	1	8.1 ± 2.7 (8)	100
Chimeric	2	8.6 ± 4.1 (7)	47
Chimeric	3	9.9 ± 4.6 (9)	42
Chimeric	4	11.5 ± 5.2 (10)	37
Chimeric	5	9.4 ± 3.8 (9)	58
Chimeric	6	19.1 ± 4.6 (9)	73
Chimeric	7	11.4 ± 5.5 (9)	33
Inbred B6	1a	4.4 ± 4.6 (10)	
F ₁ hybrid	1b	51.1 ± 3.5 (9)	
F ₁ hybrid	2b	51.1 ± 3.9 (6)	
F ₁ hybrid	3b	51.7 ± 3.4 (4)	
F ₁ hybrid	4b	53.9 ± 3.7 (4)	

Chimeric mice were used at 8 to 11 months of age.

*Data given as mean ± SD (n) where n = number of successive blood samples taken from the mouse at three- to eight-day intervals over a 47-day period. One B6 and (B6 × CBA)F₁ control were also used at each of ten intervals, and three more mice of the latter genotype (numbers 2b to 4b) were added part way through the experiment. Random losses of frozen samples caused n = 7 to 10 in the first nine mice of this Table.

seven chimeric mice over a 47-day period at intervals of three to eight days. Also shown are findings in four (B6 × CBA) F_1 hybrid mice and an inbred B6 mouse. In each chimeric mouse the proportion of ^{35}S -labeled d hemoglobin, which represents the corresponding percentage of reticulocytes of the CBA or d phenotype, was low, with mean values ranging from 8.1% to 19.1%. The F_1 hybrids showed close to 50% d hemoglobin as expected, since every reticulocyte in these mice produces hemoglobin of both the d and s types.

In contrast to the wide differences in the mean values of percent d hemoglobin between chimeric and F_1 hybrid mice, there were negligible differences in the corresponding SDs, which in the chimeric mice ranged from 2.7% to 5.5% (average, 4.4%) and in the F_1 hybrids ranged from 3.4% to 3.9% (average 3.6%). A more detailed analysis of the sources of variation was carried out using data collected at seven weekly intervals on the seven chimeric mice. The residual mean square from such an analysis excludes day-to-day variation (which is largely experimental in origin) but not within day experimental error. The corresponding SD was 4.5%, which agrees well with the average of the estimated SDs from the individual mice.

Table 2 summarizes an earlier experiment in which hemoglobin synthesis in reticulocytes from blood samples was serially tested from two chimeric mice 11 times at three-day intervals. The F_1 hybrid and B6 controls were also tested in two and seven of these runs, respectively. Percentages of d hemoglobin in the chimeric mice were low and similar to values found for the same mice three months later (Table 1). As in Table 1, the SDs in chimeric and F_1 hybrid mice were similar.

Table 3 summarizes studies in which total amounts of single and diffuse hemoglobin were serially measured by optical densities (ODs) seven to nine times at successive intervals of 60 to 136 (averaging 100) days, starting at 2 months of age in the same seven chimeric mice used in Table 1. On two occasions systematic variation occurred in the ODs such that zero readings were made for all but one mouse. These data were not used in the calculations. All seven mean values for the percent d hemoglobin were in the same range as those for freshly synthesized hemoglobin shown in Table 1; moreover, there was a moderate positive correlation between mean values obtained in the same mouse by the two assay procedures. (Spearman's $\rho = 0.57$, $p = 0.10$.) The latter finding suggests that experimental error did not obscure the consistency expected in the results of the two

Table 3. Total CBA-Type (d-Type) Hemoglobin Percentages and Other Markers in Chimeric Mice

Mouse No.	Sex	Percent CBA-Type Hb	Coat Color Type	No. CBA-Type Coat/Total Offspring	Percent CBA-Type Lymphocytes
1	M	7.6 ± 5.9 (7)	Mix	10/10	27
2	M	9.2 ± 5.3 (7)	Mix	14/16	2
3	M	7.4 ± 5.8 (7)	Mix	9/9	59
4	F	10.7 ± 6.9 (6)	Mix	1/5	29
5	F	16.2 ± 8.4 (5)	Mix	7/8	—
6	M	20.8 ± 7.3 (6)	CBA	0/0	24
7	M	14.4 ± 5.7 (5)	Mix	0/0	—

Data collections were started on chimeric mice at 8 to 10 weeks of age. Percentages of CBA-type hemoglobins are given as mean ± SD (n) where n = the number of successive blood samples taken from the mouse at 60- to 136-day (mean 100 days) intervals. Coat colors were roughly even mixtures of B6 and CBA types (mix) except in No. 6, whose coat appeared to be only CBA type. Numbers of offspring with CBA-type coat color/total offspring were measured on mating each chimera with a B6 mouse, so that the offspring would have the dominant agouti or CBA color only if this type came from the chimera. Percentages of circulating CBA-type lymphocytes were measured within three days of the last hemoglobin analysis.

assays, especially considering the low range of d hemoglobin values and small number of animals. The SD values shown in Table 3 are appreciably higher than those in Table 1 and are not correlated with the latter (Spearman's $\rho = 0.23$, $p > 0.20$), which suggests that experimental error obscured any possible agreement between a pair of values for the same animals.

Table 3 also shows that six of the chimeras had mixed CBA and B6 coat types, while one had only the CBA-type coat. All or most offspring inherited the CBA-type coat color, indicating CBA germ cells from four of the five chimeras that reproduced when tested. Percentages of CBA-type circulating lymphocytes tested after the final hemoglobin assay were higher than CBA-type (d) hemoglobin in four of five chimeras and much higher in one (Table 3).

DISCUSSION

In the chimeras we studied, the proportion of reticulocytes synthesizing CBA-type hemoglobins was subject to rather low variability that could not be distinguished from experimental error (Tables 1 and 2). The low variability was not due to dependence between values of the successive samples collected, since no systematic increase occurred in the serial correlation coefficients. These averaged -0.10 (range: $-0.43, 0.40$), which is close to the theoretical value (-0.14) associated with complete independence.²⁵ The latter finding is evidence for independence among the sequence of values observed in the individual mice; accordingly, the number of primitive stem cells that had produced reticulocyte clones (n) was estimated using the binominal formula, $n = p(1 - p)/s^2$, where p is the mean proportion of d-type hemoglobin and s^2 is the variance calculated from the series of proportions of d hemoglobin observed in a single mouse. The binomial formula can readily be derived by applying Pearson's method of moments.²⁶ The average of the numbers of primitive stem cells whose progeny actively produced reticulocyte clones

Table 2. Newly Synthesized CBA-Type (d-Type) Hemoglobin Percentages in Two Chimeric and One F_1 Hybrid Mice

Mouse Type	Identification No.	Percent Labeled CBA-Type Hb*	Calculated No. Clones
Chimeric	2	9.1 ± 3.5 (11)	68
Chimeric	4	11.7 ± 3.0 (10)	118
Inbred B6	—	3.1 ± 1.1 (2)	—
F_1 hybrid	—	49.9 ± 2.9 (7)	—

*Two of the chimeric mice described in Table 1 were used three months previously in this experiment. Blood samples were taken at 11 successive three-day intervals. Some of these data were previously published in a different form.⁴

during the three-day reticulocyte life span calculated from data in Table 1 and 2 was 64 (range, 33 to 118). This is far too high for such cells to be serially activated and inactivated, as required by clonal selection hypotheses.⁵⁻⁸

Three simulations were generated as sets of 20 random proportions from binomial distributions with n set at 10, 100, and 1,000 respectively, and $p = 0.116$ (11.6%). The first computer run was used in each case to avoid any selection, with the following results: (mean random proportions expressed as percent $\pm$ SD, estimated number of clones) 11.0 ± 8.5 , 13 clones, 11.9 ± 3.0 , 116 clones, 11.7 ± 1.2 , 717 clones. The SD of ± 3.0 associated with n set at 100 was in the range of the values we found (Tables 1 and 2).

Use of ³⁵S-methionine-labeled hemoglobin not only allowed us to estimate cell production during the short time that a circulating reticulocyte produces hemoglobin but also apparently reduced the day-to-day variability due to experimental error associated with the measurement of optical densities. On seven occasions during the short-term experiments on the chimeric mice, ODs and counts were measured on the same sample. The day-to-day variation in these data, which largely reflects experimental error, amounted to an SD of 7.6 for the ODs compared to only 2.6 for the counts. When total hemoglobins were assayed at intervals longer than mouse erythrocyte life spans, SD values ranged from 5.3 to 8.4 (Table 3). The corresponding numbers of stem cells producing erythrocyte clones estimated using the binomial formula ranged from 19 to 38; these are appreciably smaller than the estimated numbers derived from the reticulocyte data. Since the latter estimates reflect the number of primitive stem cells whose descendants were active over the short reticulocyte life span, it is impossible that they would exceed the corresponding number of stem cells active over the entire life span of the erythrocyte. We infer that experimental error associated with the measurement of ODs accounts for this discrepancy.

Most of the hemoglobin in chimeras made from the B6 and CBA strains was B6 type, consistent with results of Mintz and Palm¹⁴ but not of Warner's group,¹⁹⁻²¹ although in the former study the change to the B6 type occurred over time. Unlike other investigators, we found that the proportion of d hemoglobin in our chimeras was stable or declined only slightly over time. Although variability due to experimental error occurred in both of our assay procedures, it was small enough not to obscure the intrinsic mouse-to-mouse similar-

ity indicated by the correlation between mean proportions of CBA hemoglobin in reticulocytes studied over 47 days (Table 1) and in total hemoglobins studied over about two years in the same mice (Table 3). Consistent with reports by Warner et al,¹⁹ we found more CBA-type cells in lymphocytes than in erythrocytes in four of five chimeras studied. Coat colors were roughly equal mixtures of B6 and CBA types in six and CBA type in one of the chimeras studied, while coat colors in most of the young produced by four of five chimeras were of the CBA type, suggesting a predominance of CBA germ cells (Table 3). Thus proportions of the two types of progenitor cells in the chimeras of this study varied from tissue to tissue, as reported previously,¹²⁻¹⁵ and CBA cells did not grow as well as B6 cells in the erythroid lineage.

The data on variability in reticulocytes suggest that the number of clones active over an erythrocyte life span is at least several hundred, an estimate consistent with other recent data in mice²⁷ and in man.²⁸ These data are contradicted primarily by those of Burton et al,⁸ which have been retracted²⁷; those of Mintz et al,⁹ in which cells were artificially injected into fetal hosts; and those of Lemischka et al,¹⁰ in which descendants of individual stem cells were identified by unique retroviral inserts after transplantation into lethally irradiated recipients. Similar transplantation procedures have been shown to reduce the repopulating abilities of primitive hemopoietic stem cells 10- to 20-fold in adults.^{3,29} By comparison, a life span of normal function, or growth from fetal stages, does not detectably reduce repopulating abilities.^{2-4,29} The relatively large effects of transplantation explain why it may cause erythrocyte production by clonal mechanisms involving sequential activation of later precursors not operating in untransplanted individuals. Our data suggest that the primitive stem cells responsible for long-term erythropoiesis in unmanipulated individuals may not differentiate in terminal bursts but may function continuously. This would be consistent with our finding that the variability of hemoglobin types in repeatedly sampled chimerics was not distinguishable from that due to experimental error.

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Factor XIII A Is Synthesized and Expressed on the Surface of U937 Cells and Alveolar Macrophages

By Richard L. Kradin, Garry W. Lynch, James T. Kurnick, Mark Erikson, Robert B. Colvin, and Jan McDonagh

Factor XIII A subunit was detected in U937 cells and human alveolar macrophages by immunohistology and Western blotting. U937 cells synthesize factor XIII A subunit *de novo* under serum-free, platelet-free conditions, as indicated by ^{35}S -methionine labeling and immunoprecipitation. Thrombin-dependent activity was demonstrated to account for 98% of the total transglutaminase activity in U937 cells ($1.5 \mu\text{g}$ per 0.5×10^6 cells/mL). Intact U937 cells and alveolar macrophages and homogenates from these cells cross-linked fibrin to form γ - γ and α -polymers. Factor XIII A was detected on the surface of intact U937 cells and macro-

phages by flow cytometry and ^{125}I -labeling and immunoprecipitation. Cell surface expression of factor XIII A was augmented in the presence of several soluble macrophage activators; however, no concurrent increase in its biosynthesis was observed. The presence and cell surface expression of factor XIII A subunit within macrophages suggest new pathways by which these cells may function in clotting and in the remodeling of the extracellular matrix during inflammation and wound healing.

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SUBSTANTIAL EVIDENCE indicates that macrophages participate in extravascular clotting.¹ Macrophages synthesize coagulation factors,² express procoagulant activities,³ bind fibrin to their surface,⁴ and are a source of plasminogen activator.⁵ Factor XIII A subunit has also been demonstrated in monocytes and peritoneal macrophages.^{6,7}

Factor XIIIa catalyzes the formation of a peptide bond between certain γ -carboxamide groups of glutamine and ϵ -amino groups of lysine⁸ on fibrin and other suitable protein substrates. Factor XIII circulates in plasma as a zymogen, composed of two A subunits ($M_r = 75,000$), and two nonenzymatic B subunits ($M_r = 80,000$), with the formula, A_2B_2 .⁹ Intracellular factor XIII has been identified in platelets and megakaryocytes,^{10,11} placenta, and prostate tissue.^{12,13} Within cells, factor XIII is present exclusively as dimers of the A subunit (A_2). The activation of factor XIII requires thrombin and calcium,¹⁴ which convert both intracellular and extracellular zymogens to the same enzyme, factor XIIIa. In contrast with other tissue transglutaminases that do not require activation, factor XIII in cells exists as an enzymatically inactive zymogen.

This paper reports the biosynthesis of factor XIII A subunit in the U937 cell, a human promonocytic tumor line, that shares antigenic and functional phenotypic features with normal macrophages.¹⁵ Both the origin and localization of factor XIII in these cells is described.

MATERIALS AND METHODS

Isolation of factor XIII A and B subunit protein. Placental factor XIII A subunit was purified from Fibrogammin (kindly

provided by Behringwerke AG, Marburg, BRD),¹⁶ and the A_2B_2 and B subunits were purified from plasma as previously described.¹⁷

For electrophoresis and blotting experiments, platelets were isolated from human whole blood, anticoagulated with 3.8% citrate (1/10 vol/vol). Platelet-rich plasma (PRP) was obtained from whole blood via centrifugation at $180 \times g$ for 15 minutes, the pH was adjusted to pH 6.6, PRP was centrifuged at $2,200 \times g$ for 20 minutes, the platelet poor plasma (PPP) was removed, and the platelet pellet was resuspended in citrate buffer, pH 7.5. Following several washes in buffer, the final pellet, free of red blood cells, was resuspended in buffer, pH 7.5, containing 20 mmol/L glucose, 40 mmol/L HEPES, and 20 mmol/L glycine.

PPP was pretreated with blue Sepharose (Pharmacia, Uppsala, Sweden) to remove albumin before precipitation with 40% $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dialyzed extensively against changes of HEPES/glycine buffer, pH 7.5. PRP was treated at 56°C for ten minutes, then removed onto ice for ten minutes, and the heat-denatured fibrinogen was removed by centrifugation.

Erythrocytes were collected from red cell pellets obtained by centrifugation of whole blood at $180 \times g$. The red cell pellet was then washed in several changes of HEPES/glycine buffer pH 7.5 by repeated centrifugation.

Factor XIII-deficient plasma was obtained from a deficient individual prior to transfusion. This plasma contained no detectable factor XIII activity when examined for the incorporation of dansyl-cadaverine into casein¹⁸ or subunit A when examined immunologically.¹⁶

Assay for transglutaminase activity. Factor XIII enzymatic activity was measured by the incorporation of $1,4(n)^3\text{H}$ putrescine dihydrochloride (specific activity = 161 mCi/mg (Amersham Radiochemical Center, Arlington, IL) into Hammerstein casein (Merck, Darmstadt, BRD) as previously described.¹⁹ Nonzymogenic transglutaminase activity was assayed in the absence of thrombin. Disintegrations per minute (dpm) were measured in a β -scintillation counter.

Electrophoresis and electroblotting. Samples for electrophoresis were diluted in an equal volume of reducing (5% dithiothreitol, 8 mol/L urea, 5% sodium dodecyl sulfate [SDS]) or nonreducing (8 mol/L urea, 5% SDS) sample buffer. Samples were boiled for ten minutes, and iodoacetamide (10 mmol/L) was added. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrotransfer of proteins from polyacrylamide to nitrocellulose was performed in a Transphor Electrophoresis Unit, run at 90 V for eight hours.²⁰ BLOTTO solution containing 5% Carnation nonfat dry milk; 0.05% Tween-20; 0.01% Anti-foam A (Sigma Chemicals, St. Louis); 2 mmol/L CaCl_2 ; 0.05% NaN_3 ; and 50 mmol/L Tris, pH 8.0 was used to block active sites on the nitrocellulose paper. Blots were stained with antisera against factor XIII A or factor XIII B (Calbiochem-Behring, La Jolla, CA) for one hour at 18°C . The blots were then washed in

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several changes of phosphate buffered saline (PBS) and 0.05% Tween-20 for two hours and stained for one hour with ^{125}I -protein A (New England Nuclear, Boston). After washing, the blots were dried, and autoradiography was performed for variable intervals at -80°C with Kodak X-OMAT-AR film (Eastman Kodak, Rochester, NY).

Crosslinking of fibrin. Fibrinogen was purified from human plasma and iodinated with Na^{125}I by a lactoperoxidase method.²¹ For the fibrin crosslinking experiments, ^{125}I -fibrinogen (1 mg/mL) was crosslinked with purified placental factor XIII (20 $\mu\text{g}/\text{mL}$), CaCl_2 (6 mmol/L), and α -thrombin (10 U/mL) (a gift from Dr John Fenton, NY State Department of Health). U937 cells, U937 cell extracts (2 to 3×10^6), or alveolar macrophages (7.8×10^6 to 1×10^8) were incubated in the presence of ^{125}I -fibrinogen ($\sim 150,000$ cpm/sample), which had been pretreated with p-hydroxymercuribenzoate (PCMB) to inactivate traces of factor XIII in the fibrinogen, and factor XIII deficient plasma, CaCl_2 (20 mmol/L) and human α -thrombin (0 to 10 U/mL) for 60 to 120 minutes at 37°C . Controls of ^{125}I -fibrinogen clotted in a 0, 1/10, or 1/100 dilution of normal plasma in HEPES-glycine buffer were also analyzed. Clots were removed and washed six times in PBS with 1 mmol/L phenylmethylsulfonylfluoride followed by additional washes in HEPES-glycine buffer, pH 7.5. The washed clot was suspended in reducing buffer, dissolved by boiling for ten minutes, and analyzed by 5% or 10% SDS-PAGE. Gels were dried and developed at -80°C for autoradiography as above.

U937 culture and stimulatory conditions. U937 cells were maintained in stationary suspension cultures at a density of 1 to 2×10^5 cells/mL in RPMI 1640 (GIBCO, Grand Island, NY) in the presence of heat-inactivated, 5% fetal bovine serum (FBS) and gentamicin (50 $\mu\text{g}/\text{mL}$) in an atmosphere of 95% air and 5% CO_2 . Cells were stimulated in log-phase growth by recombinant IFN- γ (10 U/mL; Genentech, San Francisco), lipopolysaccharide (LPS; 5 $\mu\text{g}/\text{mL}$), *Escherichia coli*, 026:B6 (Sigma, St. Louis), phytohemagglutinin (PHA; 5 $\mu\text{g}/\text{mL}$; Sigma), or phorbolmyristate (PMA; 20 ng/mL; Sigma) for 72 hours at 37°C in RPMI 1640, 5% FBS and gentamicin.

Monocyte and macrophage preparation. Buffy coats were obtained from 3.8% citrated blood (1/10 vol/vol), collected in siliconized glass tubes. Peripheral blood mononuclear cells (PBM) were separated on Ficoll-Hypaque, washed three times, and resuspended in RPMI 1640 at 2×10^6 cells/mL. PBM were then allowed to adhere to glass for two hours at 37°C prior to washing in warm medium to remove nonadherent cells. Purity was assessed by the ability of adherent cells to phagocytize latex beads (Dow Diagnostics, Indianapolis) and was $>95\%$.

Human bronchioalveolar macrophages were obtained via a 300 mL-saline bronchioalveolar lavage from the right middle lobe of nonsmoking workers who had been exposed to asbestos. These patients had no evidence of lung disease by x-ray or by pulmonary function testing. The lavage was filtered through sterile gauze to remove bronchial mucus, washed and resuspended in RPMI 1640 with 5% FBS and gentamicin, and allowed to adhere to plastic or glass as described above for monocytes.

Immunohistologic staining. Control and stimulated U937 cells were washed free of medium in PBS and assessed for viability by 0.4% trypan blue exclusion. Cells (0.5 to 1.0×10^6) were distributed in polyethylene tubes (Falcon, Oxnard, CA). FcR-IgG on macrophages and U937 cells were blocked with heat-aggregated human IgG (100 $\mu\text{g}/\text{mL}$) (gift of Lynn Baird) for 30 minutes, then washed three times in ice cold PBS containing 0.3% NaN_3 . Cells were pelleted at $400 \times g$ and incubated with saturating concentrations of mouse antihuman monoclonal OKT3, which reacts with all peripheral T cells but not with U937 cells or macrophages; OKM1 (C3bi receptor), which reacts with U937 cells and all macrophages; and

rabbit antihuman factor XIII A and XIII B (Calbiochem-Behringwerke, La Jolla, CA). Cells were then washed with PBS and reacted with F(ab')_2 goat antimouse IgG-FITC (1:40) (Tago, Burlingame, CA) or F(ab')_2 goat antirabbit IgG-FITC (1:40; Cappel Labs, Downingtown, PA). Cells were washed two times in PBS, pelleted and fixed at 18°C with 2% paraformaldehyde, then washed and resuspended in PBS. Cytocentrifuge preparations of macrophages were prepared in PBS, air dried, and stained by an avidin-biotin immunoperoxidase method in a humidified chamber at 18°C . Slides were pretreated with H_2O_2 to inhibit endogenous peroxidase. The slides were incubated with primary antisera for 30 minutes, washed with PBS, and then incubated with biotin-labeled goat antirabbit IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). They were stained with 3-amino-9-ethyl carbazole and counterstained with hematoxylin.²²

Flow cytometry. Cell surface expression of factor XIII was analyzed with a fluorescence-activated flow cytometer (Spectrum III, Ortho Diagnostic Systems, Westwood, MA) after gating on the desired population utilizing their right and forward angle light scatter characteristics. Viable U937 cells form a cluster on the scatter plot in the same region as T cell blasts. The fluorescence amplifier gains were set at 520 to 600 units. These settings yielded a peak of fluorescence for glutaraldehyde-fixed erythrocytes at channel 100 using a range of 0 to 255 linear units. The median fluorescence intensity was used as a quantitative measure of the amount of binding of antibodies.

Biosynthetic labeling of U937 cells. U937 cells (6×10^6) were washed five times in methionine-free RPMI and plated in 24 well plates at 6×10^5 cells/mL for one hour. $\text{L-}^{35}\text{S}$ -methionine (120 μCi) were added to each well, and the cells were incubated for four hours at 37°C . The cells were washed three times in PBS, with 0.4% NaN_3 at 4°C . The cell pellets were washed in Tris buffer, pH 7.3, centrifuged at $4,000 \times g$, lysed, and extracted in 1 mL of Tris/NP-40 with 1 mmol/L phenylmethylsulfonyl fluoride; lysing was continued on ice in the presence of 3.5 mol/L NaCl. The lysate was precleared with Staph A (New England Enzyme Center, Boston), immunoprecipitated with rabbit antihuman factor XIII A or anti-ovalbumin for 60 minutes at 37°C , and antigen-antibody mixture was incubated with Staph A overnight. The immunoprecipitate was washed in buffer, subjected to three cycles of freezing and thawing, boiled in reducing sample buffer for ten minutes, and analyzed by 10% SDS-PAGE. The resulting gels were dried and developed in cassettes for two to four weeks with Ultrafilm (LKB, Bromma, Sweden).

Surface membrane labeling of U937 cells. U937 cells (1×10^8) were washed four times in PBS with 0.4% NaN_3 and once with 60 mmol/L phosphate buffer. The cells were suspended in 200 μL of phosphate buffer in the presence of 200 μL of lactoperoxidase, Enzymobeads (Bio-Rad, Richmond, CA), 1% beta-D-glucose, and 2 mCi of Na^{125}I for 25 minutes.²³ The reaction was quenched with iced Tris buffer, and the cell pellet was washed four times. Membranes were extracted in the presence of Lysis-EDTA buffer (0.5% SDS; 0.5% Triton; 0.23% DOC; 5 mmol/L EDTA) for 30 minutes on ice. The lysate concentrations were adjusted with 0.5 mol/L NaCl. The lysates were immunoprecipitated with rabbit antihuman factor XIII A antisera, rabbit antihuman XIII B antisera, or anti-ovalbumin IgG; suspended and boiled in reducing buffer; and analyzed on 10% SDS-PAGE. Gels were dried and developed for one to two weeks in cassettes with Kodak-X-Omat film.

RESULTS

Extraction of the factor XIII A subunit from U937 cells. NP-40 extracts from U937 cells were analyzed for factor XIII subunits after SDS-PAGE and electrotransfer

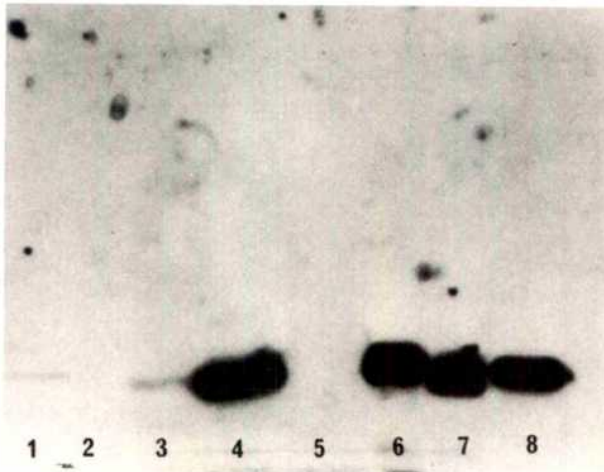


Fig 1. Antifactor XIII A subunit immunoblot of NP-40 extracts of cells. U937 cells (1×10^7 /mL) were extracted in NP-40, analyzed by 5% SDS-PAGE, immunoblotted, and stained with antibody to XIII A and ^{125} I-Protein A. Lane 1: NP-40 extract of U937 cells; lane 2: human IgG (50 μ g); lane 3: $(\text{NH}_4)_2\text{SO}_4$ precipitate of blue-Sepharose absorbed plasma; lane 4: lysate of washed human platelets (600,000/mL); lane 5: washed human red blood cell lysate (10,000/mL); lane 6: purified placental factor XIII A₂ (7.5 μ g/mL); lane 7: thrombin-treated factor XIII A₂ (autoradiograph shows reactivity with both factor XIII A subunit and its activated A' subunit); lane 8: purified A₂B₂ from plasma (15 μ g/mL).

onto nitrocellulose. The presence of factor XIII subunits was detected with monospecific rabbit antisera (Fig 1). A radiolabeled band, with $M_r = 75,000$, was seen to comigrate with purified factor XIII A subunits that had been isolated from plasma, platelet extracts, and placenta. Antifactor XIII A antisera did not crossreact with other proteins (including the B subunit of factor XIII) in plasma, platelet lysates, or U937 cell extracts. Red cell extracts, which lack factor XIII activity but are a rich source of tissue transglutaminase, failed to stain with the antisera, indicating that it did not crossreact with tissue transglutaminase. Placental factor XIII A₂, which had been cleaved by thrombin prior to electrophoresis, was identified as a second radiolabeled band with slightly smaller mol wt ($M_r = 71,000$). These experiments prove the specificity of the antisera for factor XIII A subunit. No staining of the U937 extracts were observed with antifactor XIII B antisera (not shown).

Localization of factor XIII A subunit in macrophages. Factor XIII A antigen was identified in cytocentrifuge preparations of monocytes, macrophages, U937 cells, and platelets with monospecific antisera to factor XIII A (Fig 2). Staining of factor XIII A in monocytes, U937 cells, and platelets was diffuse and homogeneous. Factor XIII A antigen in alveolar macrophages stained with variable intensity, indicating heterogeneous expression of factor XIII A antigen by these cells. Their staining was punctate and appeared to be localized to cytoplasmic organelles. Factor XIII B antigen was not detected within macrophages, platelets, or U937 cells.

To differentiate intracellular and extracellular staining, intact U937 cells and alveolar macrophages were stained in suspension and examined by fluorescence microscopy. Factor

XIII A antigen was localized along the cell surface membrane. Intracytoplasmic staining was not detected. Staining and cytofluorographic analysis of paraformaldehyde-fixed cells confirmed the presence of factor XIII A antigen on the cell surface (Fig 3). To exclude the possibility that surface staining might be due to nonspecific binding by immunoglobulin to inadequately blocked surface Fc receptors, cells were stained with other rabbit antisera, including antifactor XIII B, fibrinogen, and ovalbumin. These control antisera failed to stain the cells as judged by direct visual or cytofluorometric examination. Antifactor XIII A antibodies did not bind to other blood cells, including red cells, lymphocytes, T cell blasts, and polymorphonuclear granulocytes as judged by flow cytometry (not shown). When U937 cells were incubated in the presence of serial dilutions of purified factor XIII A (20 μ g/mL) and antiserum to XIII A, a 40% maximal decrease in the intensity of baseline surface staining for this antigen was observed. However, complete inhibition of staining was not obtained in these experiments. An identical pattern of intracellular and cell surface staining of factor XIII A antigen was observed using mouse antihuman IgM monoclonal antibodies (MoAbs) that were raised against the purified factor XIII A subunit (unpublished observations, G. Lynch and J. McDonagh).

To confirm further the identity of factor XIII A on the U937 cell surface, iodination of factor XIII A and immunoprecipitation by rabbit antibody were performed. This procedure yielded a faint radiolabeled band in the autoradiogram at ~70,000 to 80,000, consistent with the observed relative mobility of factor XIII A subunit under these electrophoretic conditions (Fig 4). This band is not more distinct because factor XIII A antibodies are conformationally dependent; and detergent, which is used for cellular extraction, can interfere with the antigen-antibody reaction. Control rabbit anti-ovalbumin did not precipitate specific radiolabeled bands. Attempts at immunoprecipitating surface factor XIII with MoAbs were unsuccessful, probably because of the IgM nature of these antibodies and conformational dependency of the antigen. Specific cell surface staining of factor XIII A has also been observed in mouse WEHI-3 and J774.2 macrophage cell lines (unpublished results, G. Lynch and J. Oliver).

Synthesis of factor XIII by U937 cells. Biosynthetic labeling of U937 cells and immunoprecipitation were performed in serum-free medium. This produced a distinct band ($M_r = 75,000$) that is consistent with the observed electrophoretic mobility of the factor XIII A subunit (Fig 5). This effectively rules out the possibility that factor XIII A expression by the cells was due exclusively to factor XIII A that had been absorbed to or internalized by macrophages from trace levels in serum. Lanes examined using the immunoprecipitated products of control antisera, ovalbumin, and anti-XIII B (not shown) displayed no specific banding.

Modulation of cell surface factor XIII A on U937 cells. Cells were examined to test if expression of factor XIII A surface-related antigen could be modulated. The intensity of U937 cell surface staining for factor XIII A antigen increased after 48 to 72 hours of incubation in media containing PMA, LPS, or IFN- γ but was unaffected by the

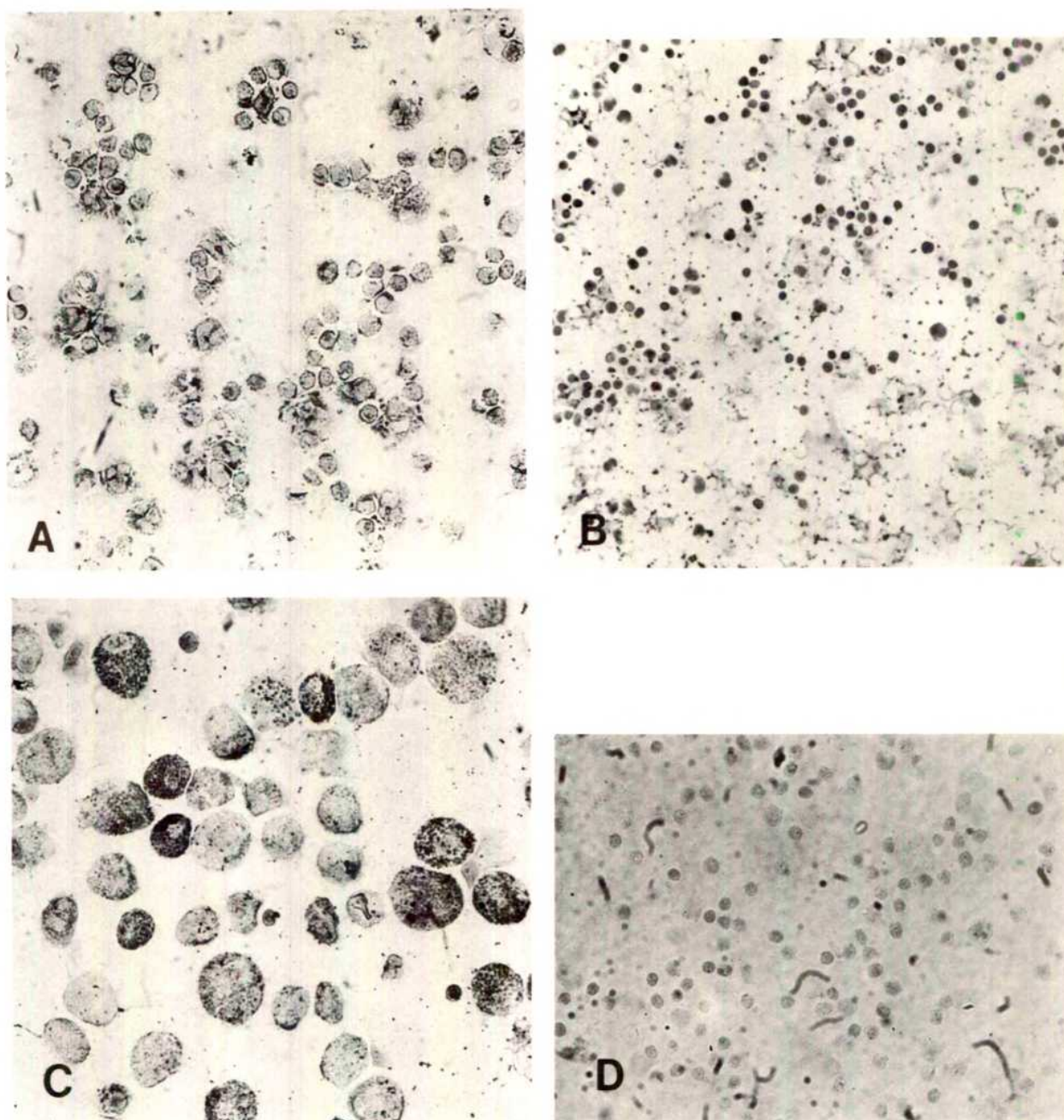


Fig 2. Identification of factor XIII A antigen in U937 cells and macrophages. U937 cells, human alveolar macrophages, blood monocytes, and platelets were prepared on glass slides by cytocentrifugation ($400 \times g$) and stained for both factor XIII A and factor XIII B subunits by an avidin-biotin immunoperoxidase method. Cytoplasmic staining for the factor XIII A subunit is present for: (A) U937 cells, magnification $\times 313$; (B) monocytes and platelets, magnification $\times 200$; (C) alveolar macrophages, magnification $\times 313$. (D) Both monocytes and platelets fail to stain for the factor B subunit, magnification $\times 200$.

presence of PHA or α -thrombin (Table 1). Expression of factor XIII B subunit was not detected under these conditions.

Factor XIII activity in macrophages. Thrombin-dependent factor XIII activity was detected in extracts of U937 cells and alveolar macrophages. Lysates of 0.5×10^6 U937 cells contained $1.4 \pm 0.1 \mu\text{g/mL}$ of thrombin-activatable transglutaminase activity. This represented $\sim 89\%$ of the

total transglutaminase activity of the cells. Thrombin-independent activity in turn accounted for $\sim 10 \pm 4\%$ of the total transglutaminase activity in U937 cells. However, if U937 cells were lysed in the presence of the serine esterase inhibitor, tosylarginylmethyl ester (TAME), thrombin-independent transglutaminase activity was reduced to $> 2\%$ of the total activity.

U937 cells cultured in the presence of $\text{IFN-}\gamma$ (10 to 100

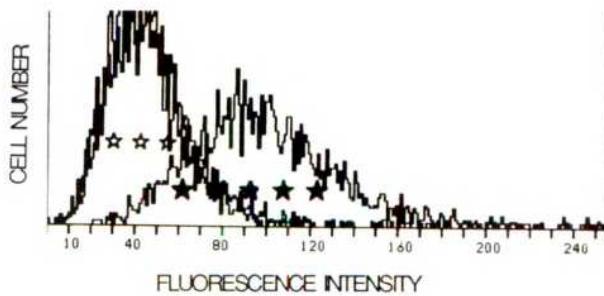


Fig 3. Flow cytometric analysis of U937 cells stained with antibodies to factor XIII A, factor XIII B, and fibrinogen. U937 cells (0.5×10^6) were washed free of medium and incubated with aggregated IgG (100 μ g/mL) before staining with antisera specific for: (A) antifactor XIII A subunit; (B) antifactor XIII B subunit; or (C) antifibrinogen for 30 minutes at 4 °C. Cells were washed and stained with F(ab')₂ goat antirabbit-IgG-FITC. The cells were analyzed on an Ortho Spectrum III cytofluorometer at standardized fluorescent gains. Staining histograms from all three conditions are represented. Vertical axis = cell number; horizontal axis = fluorescence intensity. Note the positive fluorescence for the factor XIII A subunit (filled stars). Curves representing the staining results for factor XIII B subunit and fibrinogen are superimposed (open stars) and failed to stain above the level for the second antibody control [F(ab')₂ goat antirabbit IgG-FITC] alone.

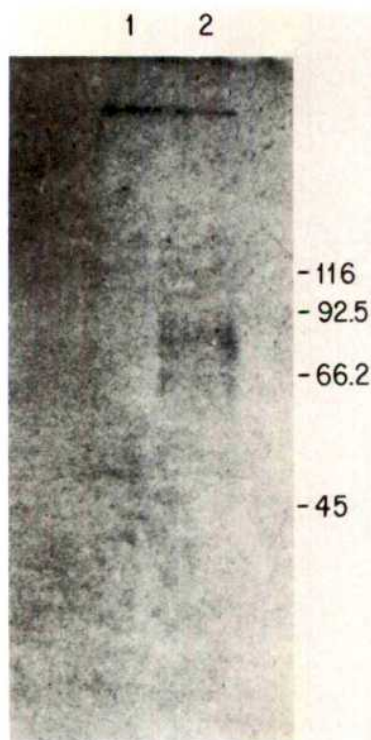


Fig 4. ¹²⁵I surface labeling of U937 and immunoprecipitation of factor XIII A. U937 cells (1×10^6) were labeled with Na¹²⁵I by a solid phase lactoperoxidase technique. The labeled membranes were extracted with detergent, immunoprecipitated with antifactor XIII A and anti-ovalbumin, and analyzed by 10% SDS-PAGE. Lane 1 antihuman ovalbumin; lane 2, antifactor XIII A.

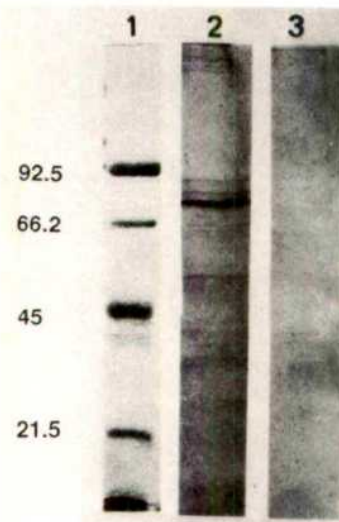


Fig 5. ³⁶S-methionine biosynthetic labeling of U937 cells. U937 cells (6×10^6) were labeled in vitro by ³⁶S-methionine in methionine-free, serum-free media. Supernatants and extracts were precleared with Staphylococcus A and immunoprecipitated. The samples were boiled in reducing sample buffer and analyzed by 10% SDS-PAGE. Autoradiographs of the dried gels were produced on LKB Ultrafilm. Lane 1: mol wt markers; lane 2: antifactor XIII A; lane 3: anti-ovalbumin. Antifactor XIII B was also negative.

U/mL) for 18 to 48 hours showed no measurable increase in cell-related transglutaminase activity, nor was enzymatic activity detected in the external conditioned medium (Table 2). Factor XIII activity in U937 did not increase after similar times in culture in the presence of concanavalin A (Con A), PHA-induced human T cell lymphokine, LPS, and α -thrombin. It appears, therefore, that factors that can increase the surface expression of factor XIII A antigen (Table 1) may not increase the total factor XIII enzymatic

Table 1. Modulation of Surface Expression of Factor XIII A Antigen

Activator/Antigen	T3	Median Fluorescence Intensity		
		C3biR	XIII A	XIII B
Control	23	54	58	45
LPS (5 μ g/mL)	28	61	96	42
PMA (20 ng/mL)	23	92	96	42
IFN- γ (10 U/mL)	24	90	90	43
PHA (5 μ g/mL)	24	78	58	45

U937 cells were maintained in stationary culture at 1 to 2×10^5 cells/mL for 24 to 72 hours in the presence of various activators. Cells were washed, Fc receptors were blocked with aggregated IgG (100 μ g/mL), and stained in suspension by an indirect fluorescence technique. T3 and C3bi receptor antigens were stained with F(ab')₂ goat antimouse IgG-FITC. Factor XIII A and factor XIII B subunits were stained with F(ab')₂ goat antirabbit IgG-FITC. Cell staining, after 72 hours of incubation, was analyzed by flow cytometry. Data represent the median fluorescence intensity of the gated cell population for each condition. Note the increase in expression of factor XIII A antigen after stimulation with LPS, PMA, γ -IFN but not PHA. The C3bi receptor antigen increases with exposure to PMA, γ -IFN, and PHA but is minimally affected by LPS.

Table 2. Transglutaminase Activity of U937 Cells $\pm$ Stimulation by IFN- γ

0.5 $\times$ 10 ⁶ U937 cells/mL	Assay	³ H-Putrescine (dpm)
RPMI	-Thrombin	108 $\pm$ 187
	+Thrombin	1129 $\pm$ 36
IFN- γ	-Thrombin	82 $\pm$ 103
	+Thrombin	1117 $\pm$ 121

U937 cells were grown in serum free RPMI $\pm$ IFN- γ (10 U/mL) for 24 hours. The cells were washed in HEPES-glycine buffer, pH 7.5, resuspended to 0.5 $\times$ 10⁶ cells/mL, and lysed by sonification. Assays were performed in the presence and absence of α -thrombin. Values show the mean and SDs of four separate determinations in disintegrations per minute (dpm) of ³H-putrescine incorporation into casein at pH 7.5.

activity of the cell. Alternatively, increased synthesis of factor XIII A may be offset by its increased intracellular degradation.

Macrophage-induced crosslinking of fibrin. U937 and alveolar macrophage cellular extracts or nonextracted U937 cells and alveolar macrophages incubated in the presence of factor XIII-deficient plasma, PCMB-treated ¹²⁵I-fibrinogen, thrombin, and calcium were capable of producing γ - γ crosslinks and α -polymers in fibrin, but lanes analyzed in the absence of cells or their extracts failed to display crosslinking (Fig 6). Lanes containing cell extracts displayed degradation of the α -chain of fibrin. This phenomenon of α -chain degradation and γ - γ crosslinking has also been observed in systems in which platelets were the only source of factor XIII.²⁴

Intact U937 cells and alveolar macrophages were pretreated with human α -thrombin (0.1 to 10 U/mL), washed, and exposed to PCMB-treated ¹²⁵I-fibrinogen in the presence of calcium, without additional thrombin. Supernatants analyzed by SDS-PAGE revealed the presence of labeled γ - γ dimers. Analysis of the gels by laser densitometry showed a progressive increase (0.5% to 14% of total protein per lane) in γ - γ dimer formation with the concentration of α -thrombin used to treat the intact U937 cells. Similar results were obtained for human alveolar macrophages (data not shown).

DISCUSSION

These studies show that macrophages synthesize factor XIII A subunit *de novo* and are a source of factor XIII activity. With the U937 cell as a model, biosynthesis of factor XIII A occurs in serum-free, platelet-free conditions. Furthermore, macrophage stimulation may be accompanied by translocation of factor XIII A from within the cytoplasm to the cell surface. These findings serve to expand the potential repertoire of macrophages in interactions with the clotting system and in the remodeling of the extracellular matrix that occurs following tissue injury.

Some previous studies have suggested that tissue transglutaminase is the dominant, if not sole source, of transglutaminase activity in macrophages,²⁵⁻²⁷ while other studies find factor XIII in macrophages.^{6,7} One reason for this discrepancy may be that factor XIII zymogen can only be isolated from cells in the presence of EDTA and without reducing conditions. Factor XIII is distinct from tissue transglutaminase by virtue of its immunochemistry, dependence on



Fig 6. Crosslinking of fibrin by U937 cells and alveolar macrophages. Purified PCMB-fibrinogen or fibrinogen in plasma and ¹²⁵I-fibrinogen were clotted with calcium (6 mmol/L) and thrombin (10 U/mL) under varying conditions. The clots were washed, boiled in sample buffer, and analyzed by 10% SDS-PAGE under reduced conditions. Lane 1: fibrin control; lane 2: fibrin and purified placental factor XIII (20 µg/mL); lane 4: fibrin and alveolar macrophage cell lysate (8 $\times$ 10⁶ cells/mL); lane 5: platelet poor plasma; lane 6: 1/10 dilution of platelet poor plasma; lane 7: 1/100 dilution of platelet poor plasma; lane 8: factor XIII-deficient plasma and U937 cell lysate (1 $\times$ 10⁶ /mL). The source of unlabeled fibrinogen was PCMB-fibrinogen in sample lanes 1 to 4 and plasma in lanes 5 to 8. The formation of both γ - γ dimers and α -polymers was observed in the presence of cell extracts without extraneous factor XIII. α -Chain proteolysis (α) was most evident in lanes that contained cell extracts.

thrombin for activation, and its mode of crosslinking fibrin and other substances.^{12,28,29} In this study thrombin-dependent, factor XIII-like activity, assayed in the presence of TAME, accounted for ~98% of the transglutaminase activity within the U937 cell line. This decrease in non-thrombin-related activity in the presence of TAME (from 10% to >2%) may result from the presence of cathepsin-like acid proteases in macrophages, which can activate factor XIII in platelets and are inhibitable by TAME (unpublished observation, G. Lynch). U937 cells are free of surface contamination by platelets. This fact serves effectively to exclude platelets as the source of factor XIII in these experiments and further supports previous observations that factor XIII is present in macrophages.^{6,7}

Factor XIII A antigen was detected on the cell surface membrane of macrophages. Most cysteine metalloenzymes are located within the cytoplasm of cells, making the presence of factor XIII on the surface of macrophages apparently unique. Only translocation of factor XIII A appeared to be affected by macrophage stimulation; factor XIII A secretion was not affected. The expression of cell surface factor XIII A was augmented under conditions that favor expression of other macrophage-associated clotting proteins. LPS is the prime stimulus to the production of procoagulant activity by

macrophages.³⁰ PMA and IFN- γ display a broad array of macrophage activating properties, including promotion of cellular attachment and spreading on most surfaces, superoxide release, cytotoxicity for tumor targets, and increased expression of macrophage fibronectin receptors.³¹⁻³³ Both PMA and IFN- γ promote an increase in the intracytoplasmic concentration of protein kinase-C. PMA has also been shown to produce translocation of intracytoplasmic protein kinase-C into the cell surface membrane.³⁴

Crosslinking of fibrin is a principal role of plasma factor XIII in hemostasis. This is an orderly process that proceeds stepwise via thrombin and calcium-dependent activation of the active site on the factor XIII A subunit and results in the formation of γ and α -crosslinkages with fibrin. This reaction, which occurs readily for fibrin, also takes place at slower rates, when fibrinogen is used as substrate, with α -chain polymerization being faster than γ -chain dimerization.³⁵ With fibrin as a substrate, γ -chain dimers form more rapidly than α -polymers. Crosslinking of fibrin renders the clot highly insoluble and alters the elastic modulus of the clot.³⁶ Also, by covalently incorporating α_2 -plasmin inhibitor into the clot, factor XIII renders fibrin resistant to plasmin-mediated proteolysis.³⁷

In addition to its role in crosslinking fibrin, factor XIII can crosslink several other matrix proteins that are present in vivo in the inflammatory reaction. After fibrin, fibronectin constitutes the next most abundant protein in the plasma clot. Crosslinking of fibrin to fibronectin occurs between the aminoterminal of fibronectin and the α -chain of fibrin.³⁸ Fibronectin can be crosslinked to most types of collagen except type IV.³⁹ Factor XIII also crosslinks fibrin to von Willebrand factor (vWF)⁴⁰ and vWF to collagen.⁴¹ Thrombospondin is a substrate for factor XIII crosslinking.⁴² Gel-solin has recently been shown to be a substrate for factor XIII, and it is also incorporated by crosslinking into plasma clots.⁴³

The potential physiologic consequences of these reactions for macrophages are profound. Macrophages bind fibrin and fibronectin to their surface and secrete thrombospondin.⁴⁴

They display increased adherence and spreading on surfaces coated with fibrin, fibronectin, and collagens. Monocytes increase their expression of Fc and C3b receptors, secrete proteases, interleukin-1, and growth factors following adherence to matrix proteins that are substrates for factor XIII.⁴⁵⁻⁴⁷

The expression of factor XIII represents a potential mode by which macrophages may interact directly with the extracellular matrix. Macrophages and exudative monocytes are likely to provide a local source of factor XIII in inflamed tissues. By stabilizing the extravascular clot to plasmin digestion, factor XIII may favor the persistence of the fibrin network at sites of injury. This clot matrix may provide an attachment site for other macrophages, fibroblasts, and endothelium during inflammation. Clark and Colvin⁴⁵ and Colvin⁴⁸ have shown that fibrin and fibronectin provide a provisional matrix for cell attachment and migration following wounding and prior to the appearance of laminin and Type IV collagen. The capacity of factor XIII to crosslink fibronectin and vWF to fibrin and to collagens suggests that macrophages may play an important role in mediating the order of the remodeling process. A role for macrophages in wound healing is supported by the observation that healing is delayed in their absence.⁴⁹ In addition to suffering from hemorrhagic diatheses and spontaneous abortions, ~14% of factor XIII-deficient individuals display defective wound healing.⁵⁰ However, it is not yet known whether inflammation or the immune response in these patients is altered or whether they are specifically deficient in macrophage-related factor XIII. Clearly, further studies are indicated to define the role of macrophage-associated factor XIII in vivo.

ACKNOWLEDGEMENT

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Thrombocytopenia Associated With Pregnancy in a Patient With Type IIB von Willebrand's Disease

By Margaret E. Rick, Sybil B. Williams, Ronald A. Sacher, and Laurie P. McKeown

Thrombocytopenia may accompany variant (type IIB) von Willebrand's disease (vWD) and is thought to result from binding of the abnormal von Willebrand factor (vWF) to the patient's platelets with subsequent platelet aggregate formation and clearance. We have studied a patient with type IIB vWD who became thrombocytopenic during two pregnancies. During the third trimester of pregnancy, her platelet counts dropped to 20,000 to 30,000/ μ L, and an increase in the intermediate-sized vWF multimers was seen on agarose gel electrophoresis. During this time her platelet-rich plasma showed spontaneous platelet aggregation, and her plasma caused spontaneous aggregation of normal washed platelets. Antibody to platelet glycoprotein Ib completely blocked the spontaneous platelet aggrega-

tion, while antibody to platelet glycoprotein IIb/IIIa did not block the response at the concentrations used. Inhibitors of platelet function that elevate platelet cyclic AMP also blocked the response, but aspirin had no effect on the spontaneous platelet aggregation. The patient illustrates that the platelet counts in one individual can vary greatly in type IIB vWD and that the thrombocytopenia that occurs can appear under physiologic conditions that stimulate the endogenous production of the patient's abnormal vWF. The mechanisms leading to spontaneous platelet aggregation and thrombocytopenia appear to be similar to those described for other patients with type IIB vWD.

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VON WILLEBRAND'S DISEASE (vWD) is an autosomally inherited bleeding disorder in which the von Willebrand factor (vWf) is quantitatively decreased and/or is qualitatively abnormal, leading to defects in platelet-subendothelial interaction and in platelet-platelet interaction.^{1,2} Variant types of vWD have been defined by the presence of an abnormal multimeric structure of the vWf protein and in some types by increased binding of the abnormal vWf to platelets when low concentrations of ristocetin are present. These variants are called type II vWD and are further categorized: in type IIB, the patient's plasma vWf lacks the largest multimers, while the patient's platelets contain the full spectrum of multimers; additionally, the abnormal vWf binds more avidly to platelets in the presence of low concentrations of ristocetin and causes platelet agglutination.^{3,4} Several patients with type IIB vWD have been described who have associated thrombocytopenia, postulated to be a result of in vivo binding of their abnormal vWf to their platelets followed by the spontaneous formation of platelet aggregates and clearance.^{5,6}

We have studied a patient with type IIB vWD during two pregnancies who is unusual because she became thrombocytopenic only during the pregnancies. Investigation of this patient's plasma obtained during the third trimester of pregnancy when she was thrombocytopenic showed that her platelets aggregated spontaneously and that her platelet-poor plasma caused aggregation of normal platelets.

Increased quantities of intermediate and large-sized vWf multimers were also observed on agarose gel electrophoresis of her plasma during this time. These phenomena were not observed before or after the gestational periods. This patient provides insight into the pathophysiology of the thrombocytopenia in type IIB vWD; she represents a IIB variant who becomes thrombocytopenic only during periods of increased production of her abnormal vWf, in this case brought about by the physiologic stimulus of pregnancy. It also demonstrates that nonthrombocytopenic patients with type IIB vWD can produce and release larger multimers of vWf and that these may cause significant thrombocytopenia. The recognition of this syndrome is important because of the difficult clinical management problems associated with it. This patient had marked blood loss during the delivery of her first pregnancy, a stillbirth, and she required Cesarean section and the use of both cryoprecipitate and platelets during her second delivery; she had excessive blood loss in spite of these measures.

CASE REPORT

This 33-year-old white female was initially referred to the National Institutes of Health in 1981 during the ninth month of her pregnancy because of recently discovered thrombocytopenia and a life-long history of bruising. She had recurrent epistaxis during both childhood and adult years, bled with loss of deciduous teeth, and had heavy menstrual bleeding. Her father and paternal grandfather also had a history of life-long epistaxis and easy bruising. The patient's obstetrician had noted thrombocytopenia during the sixth month of her pregnancy but referred her to a hematologist only when the platelet count was noted to drop to <50,000 during the eighth month. A bone marrow aspiration showed normal numbers of megakaryocytes, a presumptive diagnosis of idiopathic thrombocytopenic purpura was made, and the patient was placed on prednisone, 60 mg/d when her platelets dropped to 20,000 to 30,000/ μ L. The patient was referred to NIH for further evaluation because of the lack of response in her platelet count while on prednisone.

Physical examination showed fading ecchymoses on all extremities and findings compatible with a normal pregnancy in the ninth month. The patient's laboratory data are shown in Fig 1 (12/23/81). Spontaneous labor began within one week of the expected date of delivery; although no fetal heart sounds were detected on admission to her private hospital, a prolonged labor was allowed to proceed terminating with the vaginal delivery of a dead fetus on 1/5/82. The

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Date	vWf:Ag (%)	Ristocetin Cofactor (%)	VIII:C (%)	Platelet Count (per μ L)	Bleeding Time (min)	vWf Multimer Pattern
12/23/81	155	95	100	41,000	19	
8/18/82	45	13	40	147,000	14	
8/24/83	55	7	56	237,000		
1/29/85	56	54	80	61,000		
3/19/85	70	51	—	49,000		
4/14/85	82	60	56	40,000		
8/23/85	33	19	68	216,000		
Normal range	55-168	59-143	50-200	143-365,000	2-9	

Fig 1. Patient's laboratory data; the vWf multimer gels are shown on the right with top of gel at left, bottom at right.

delivery was complicated by hypertension, a third-degree perineal laceration and subsequent infection, and marked blood loss requiring 65 units of blood products, including RBCs, whole blood, platelets, and cryoprecipitate. The prednisone was slowly discontinued over the next four months, and the patient's platelets gradually returned to normal during that time.

The patient remained stable with normal platelet counts on multiple samples during the next two years (Fig 1). Studies of her vWf and factor VIII (VIII:C) were performed, which confirmed the diagnosis of type IIB vWd (Fig 1). She became pregnant again in the fall of 1984 and had a normal pregnancy except that thrombocytopenia was noted to develop during the second trimester of pregnancy and persisted through the third trimester (Fig 1); spontaneous labor began on April 14, 1985, and a healthy boy was delivered at Georgetown University Hospital by a planned cesarean section. The patient was given both cryoprecipitate and platelets prior to and following delivery but had increased blood loss of approximately 1,800 cc. She recovered uneventfully.

METHODS

The patient gave informed consent for the studies. Blood was obtained by a two-syringe technique using a 19-gauge needle and was placed in 1/100 volume of sodium citrate (10.9 mol/L final concentration). Platelet-poor plasma was obtained by centrifugation at 2,000 g for ten minutes at 4°C; the plasma was used immediately or was frozen in aliquots at -70°C.

Platelet-rich plasma for aggregation studies was obtained by centrifugation of citrated whole blood at 750 g for three minutes at room temperature; the platelet count was adjusted to 200,000/ μ L with platelet-poor plasma (or less as noted in each experiment when the patient was thrombocytopenic; in that case the normal control was adjusted to the same platelet count). Platelet aggregation was completed within two hours using a Chrono-Log Lumi Aggregometer (Chrono-Log, Havertown, PA). Baseline values for light transmission in platelet-rich plasma were adjusted to 5% to 10% on the recorder. Agonists for routine platelet aggregation studies included epinephrine, 7.5 μ mol/L (Parke Davis, Morris Plains, NJ); ADP, 3.8 μ mol/L (Sigma, St. Louis); collagen, 225 μ mol/L (Millipore, Freehold, NJ); and ristocetin, (Lenau, Copenhagen, Denmark). Ristocetin-induced platelet aggregation (RIPA) was tested by adding ristocetin at final concentrations varying from 0.34 to 2.0 mg/mL to 400 μ L of platelet-rich plasma; a normal control was run alternately with the patient sample.

Spontaneous platelet aggregation was assessed by placing 400 μ L platelet-rich plasma in an aggregometer cuvette, stirring at 900 rpm and following the change in light transmission for 15 minutes; control platelet-rich plasma prepared in parallel was alternated with the patient's sample during the testing. When antibodies (purified

IgG) or inhibitors were used in the platelet aggregation studies, they were added to the platelet-rich plasma three minutes before addition of the stir bar (except P-PACK, which was added ten minutes before). Two monoclonal antibodies (MoAbs) were used, both the gift of Dr Barry Coller: 6D1 directed against platelet glycoprotein Ib (GP Ib) (9.8 μ g/mL final concentration), and 10E5 directed against platelet glycoprotein IIb/IIIa (GP IIb/IIIa) (1 to 16 μ g/mL final concentration). The inhibitors included PGI₂, 24 μ g/mL (Sigma Chemical); db cAMP, 9.1 mmol/L (Sigma); acetyl salicylic acid (ASA), 0.45 μ g/mL (Aldrich Chemical, Milwaukee); and D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone (P-PACK), 10⁻⁶ mol/L (Calbiochem-Behring, LaJolla, CA), all given as final concentrations.

Mixing studies were performed using platelets that were separated from plasma proteins by arabinogalactan gradient centrifugation as previously reported.⁷ Platelets from a normal subject who had not taken medications for two weeks and platelets from the patient were prepared simultaneously. In these experiments the separated platelets and platelet-poor plasma were mixed in the cuvette to a final concentration of 200,000 platelets/ μ L in a total of 0.4 mL volume. Spontaneous platelet aggregation was assessed as noted above. In instances where an inhibitor or antibody was added, the separated platelets and platelet-poor plasma were placed in the cuvette and the inhibitor added immediately and incubated for three minutes before stirring and platelet aggregation.

VIII:C, vWf antigen (vWf:Ag) and ristocetin cofactor assays were performed as previously reported.^{8,9} One unit of activity or antigen is that present in 1 mL of pooled normal plasma for each assay. Glyoxyl agarose gel electrophoresis was performed according to the method of Hoyer and Shainoff.¹⁰

Bleeding times were performed by the Mielke et al template method.¹¹

RESULTS

Coagulation studies. When initially seen during the ninth month of her first pregnancy, the patient was thrombocytopenic and had a prolonged bleeding time of 19 minutes (Fig 1). Her factor VIII and vWf values were within the normal laboratory ranges (nonpregnant population), but her vWf multimer pattern showed a decrease in the largest multimers (Fig 1: 12/23/81). After recovery from the traumatic delivery and perineal infection, the patient's platelet count rose to the normal range and her factor VIII and vWf values fell to abnormal levels; the vWf multimer pattern became more distinctly abnormal, with an overall decrease in quantity and fewer large forms present (Fig 1: 8/82 to 8/83). During her second pregnancy, decreased platelet counts, increased factor VIII and vWf values, and vWf multimer patterns similar to those seen in the first pregnancy were observed (Fig 1: 1/29/85 to 4/14/85); these values returned to the patient's baseline levels within approximately one month after delivery.

Platelet aggregation studies. Routine platelet aggregation studies performed when the patient was not pregnant and when her platelet count was in the normal range showed normal aggregation responses and release of ATP to epinephrine, ADP, and collagen. Ristocetin-induced platelet aggregation revealed that the patient's platelets responded to 0.34 mg/mL of ristocetin, while control platelet-rich plasma required >0.87 mg/mL to elicit an aggregation response (Table 1). No spontaneous platelet aggregation was observed when the patient was not pregnant.

Table 1. RIPA: Initial Slopes of Patient and Control at Varying Ristocetin Concentrations

Ristocetin (mg/mL)	Patient (mm/min)	*Control (mm/min)
1.30	40	47
0.87	31	3
0.52	14	0
0.34	8	0

Platelet-rich plasma from patient and control (200,000/ μ L) were run alternately using the concentrations of ristocetin noted.

*Average for PRP from six normal subjects: 0.34 mg/mL, 0 mm/min; 0.5 mg/mL, 4 mm/min; 1.0 mg/mL, 10 mm/min; 2.0 mg/mL, 62 mm/min.

Special platelet aggregation studies. Studies performed during the third trimester of the patient's second pregnancy demonstrated that the patient's platelets aggregated spontaneously (Fig 2, panel A). This spontaneous aggregation was inhibited by the MoAb to GP Ib; however, the antibody to GP IIb/IIIa did not inhibit aggregation (Fig 2, panels B and C).

Mixing studies using platelet-poor plasma and platelets separated from plasma proteins indicated that the patient's plasma rather than her platelets was responsible for the spontaneous platelet aggregation. Patient plasma added to normal platelets showed a spontaneous aggregation response (Fig 2, panel D), while normal plasma added to patient platelets showed no spontaneous aggregation. The aggrega-

tion response observed with patient plasma and normal platelets could be inhibited by the antibody to GP Ib but could not be inhibited by the antibody to GP IIb/IIIa, similar to that seen with patient platelet-rich plasma.

The addition of various inhibitors to the patient's platelet-rich plasma revealed no inhibitory action of ASA; however, PGI₂ caused disaggregation of an early small aggregation response, and the thrombin inhibitor P-PACK abolished the spontaneous aggregation. Prostaglandin I₂ and dibutyryl cyclic AMP also inhibited the spontaneous aggregation in the mixture of patient plasma and normal platelets (data not shown).

Studies performed four months after the successful termination of her second pregnancy showed that the patient no longer demonstrated spontaneous platelet aggregation. Ristocetin-induced platelet aggregation (1.3 mg/mL) of patient platelet-rich plasma was completely abolished by preincubation with the anti-GP Ib but not by the anti GP-IIb/IIIa, similar to that seen with normal platelet-rich plasma. The patient, but not the control, aggregated when ristocetin was added at a final concentration of 0.87 mg/mL; the anti-GP IIb/IIIa abolished this aggregation response. This antibody also prevented aggregation of patient platelets when ADP (5, 10, and 25 μ mol/L) was added.

DISCUSSION

Recent studies have indicated that the abnormal vWf of patients with type IIB vWd binds to the platelet membrane receptor GP Ib in the absence of ristocetin. This binding apparently stimulates exposure of the platelet membrane receptor for fibrinogen, GP IIb/IIIa, making it available to plasma fibrinogen, which binds and initiates aggregation.^{5,12} The platelet aggregates are then thought to be cleared *in vivo* by normal clearance processes. Although the majority of patients with type IIB vWd do not have thrombocytopenia, a number of patients have been reported with low platelet counts.^{5,6} Thrombocytopenia has also occurred in type IIB patients after therapeutic infusion of desmopressin (DDAVP), which transiently elevates levels of vWf in the circulation, including the largest multimers, making them available to bind to platelets.¹³

The production of an abnormal vWf in our patient was physiologically stimulated during pregnancy, and the levels rose into the "normal" range; larger multimers than had been previously noted were also observed. Both the qualitative and quantitative changes in the abnormal vWf led to spontaneous platelet aggregation and thrombocytopenia, likely by the mechanism outlined above. Although this same phenomenon may occur when the patient is not pregnant, it appears that the concentration of abnormal vWf is not sufficient to lead to enough platelet aggregation *in vivo* to cause thrombocytopenia. The levels of abnormal vWf and the larger multimers in particular are clearly important in the clinical manifestation of thrombocytopenia.

Antibodies to GP Ib completely inhibited the spontaneous aggregation in our patient, similar to previous studies.^{5,12} The anti-GP IIb/IIIa did not inhibit the aggregation, however. Since this concentration of anti-GP IIb/IIIa did prevent the exaggerated response to ristocetin (0.87 mg/mL) and pre-

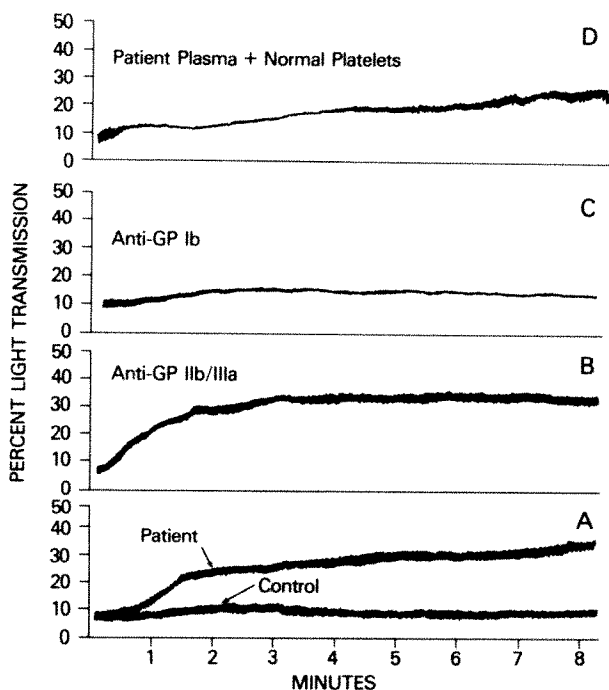


Fig 2. Spontaneous platelet aggregation: panel A, spontaneous platelet aggregation of patient's platelet-rich plasma with normal platelet-rich plasma control; panels B and C, inhibition of spontaneous platelet aggregation in patient's platelet-rich plasma by anti-GP Ib (panel C) and lack of inhibition by anti-GP IIb/IIIa (panel B); panel D, spontaneous platelet aggregation of isolated normal platelets induced by patient's plasma.

vented ADP-induced platelet aggregation four months after delivery, there may have been another mechanism (not related to GP IIB/IIIa) mediating part of the spontaneous platelet aggregation during pregnancy. Alternatively, the lack of inhibition by anti-GP IIB/IIIa may be related to the quantity of the antibody required to prevent exposure of the fibrinogen binding site during pregnancy in this patient despite the fact that this quantity of 10E5 has prevented spontaneous platelet aggregation in several other (nonpregnant) type IIB patients.⁵

The spontaneous platelet aggregation in this patient was inhibited by agents that increase platelet levels of cyclic AMP (PGI₂ and db cAMP) but was not inhibited by aspirin, indicating that the aggregation is not dependent on prostaglandin metabolism. This pattern of inhibition has been observed previously in patients with type IIB vWd.⁵ The inhibition by P-PACK suggests that generation of small amounts of thrombin may have contributed to the exposure of fibrinogen binding sites in this patient; however, hirudin did not show any inhibitory effect in this patient's spontaneous platelet aggregation (data not shown), so the mechanism of action of P-PACK in this setting is not clear.

This patient demonstrates that there can be a wide spectrum of platelet counts and variable decreases in the larger vWf multimers within one patient with type IIB vWD, depending on the physiologic conditions. The patient further

suggests that the presence or absence of thrombocytopenia is related to the level of circulating endogenous larger vWf multimers. A family recently reported¹⁴ with a normal baseline vWf multimer pattern on agarose gels and increased RIPA at low concentrations of ristocetin may also represent part of this spectrum. In that family, the addition of low concentrations of ristocetin to the patients' PRP caused binding of the largest vWf multimers, resulting in a residual type IIB multimer pattern. While the molecular defect(s) is unknown in type IIB vWd patients and in this family, it seems likely that the defect(s) leads to an increased ability of the abnormal vWf to bind to GP Ib.

Clinical evaluation of pregnant patients who present with thrombocytopenia should include a careful questioning about life-long bleeding problems and any family history of excessive bleeding; if the history is positive, evaluation of the patient's vWf, including multimer studies, should be carried out. The clinical management of pregnancy in patients with type IIB vWD requires close clinical and laboratory supervision and conservative treatment with cryoprecipitate and platelets.

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Initial Experience in Treating Human Lymphoma With a Chimeric Univalent Derivative of Monoclonal Anti-idiotypic Antibody

By T.J. Hamblin, A.R. Cattan, M.J. Glennie, M.R. MacKenzie, F.K. Stevenson, H.F. Watts, and G.T. Stevenson

Murine monoclonal anti-idiotypic antibody was raised against the surface IgM on the neoplastic cells of a patient with widespread follicular lymphoma. For therapy, a chimeric antibody derivative, FabIgG, was constructed by thioether-linking Fab γ , from the monoclonal antibody, to human normal IgG. FabIgG is univalent and thereby avoids rapid antigenic modulation. Its human IgG component is intended to optimize recruitment of effectors and metabolic survival while minimizing immunogenicity. Four intravenous (IV) infusions of 380 to 580 mg of anti-idiotypic FabIgG were given over a period of 11 weeks. There was no significant toxicity. On each occasion, the antibody disappeared from the plasma with a half-life ($t_{1/2}$) of <24 hours. The brief survival was evidently due to uptake by tumor, as infused control FabIgG, containing Fab γ from an

irrelevant antibody, yielded a plasma $t_{1/2}$ of >10 days. With each therapeutic infusion, there was a fall in the number of circulating neoplastic cells over a 24-hour period. The numbers were largely replenished over the next week, but a net fall became discernible over the entire period of treatment. Four days after each infusion, nodal masses were swollen and tender, subsiding over ~8 days. At the end of the treatments, the blood lymphocyte count and nodal and splenic swellings continued to subside, so that by 6 weeks a partial remission with removal of >50% of tumor was judged to have occurred. We did not detect any qualitative change in surface idiotype nor any antibody response to the infused Ig.

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THE TREATMENT OF lymphoid malignancies with antibody has to date yielded quite modest results, with most patients showing only partial or transient remissions.¹ Included in this experience is the use of anti-idiotypic antibody (anti-Id) directed against variable regions of tumor surface immunoglobulin (Ig).²⁻⁷ The target represents a uniquely well-characterized differentiation antigen, of specificity so narrow that at present an individual anti-Id must be raised for each tumor to be attacked.⁸ Monoclonal technology has improved therapeutic prospects by making anti-Id available as a homogeneous reagent in indefinitely large quantities but, despite initial promise,⁴ its inherent antitumor effect is unlikely to be better than that of earlier polyclonal reagents when given in unmodified form.

Factors limiting the therapeutic efficacy of anti-Id are by no means fully defined.^{1,8} They probably include sparse surface Ig, an appreciable secretion of the tumor Ig, idiotypic heterogeneity within a tumor, antigenic modulation, the failure of antibody to recruit appropriate effectors (the roles of complement and the various cellular effectors are poorly understood), and induction of an anti-antibody immune response. Some of these factors can be taken into account when cases suitable for therapy are selected, and some can be by designing suitable derivatives of antibody. For example,

experience has emphasized the difficulties involved in treating with anti-Id those lymphomas secreting appreciable idiotypic Ig into extracellular fluid⁶; we showed in animal lymphoma that certain univalent antibody derivatives, which avoid antigenic modulation, are therapeutically superior to the bivalent parent antibodies.^{9,10}

In this article, we present initial data on the therapeutic use in humans of an antibody derivative designed to avoid or minimize several of the above problems. The derivative is the chimeric univalent antibody, FabIgG (Fig 1). It consists of Fab γ from mouse monoclonal anti-Id, linked by tandem thioether bonds to human normal IgG. Preparation and preliminary characterization of FabIgG have been described.¹¹ Being univalent, it avoids rapid antigenic modulation.^{12,13} The possession of a human Fc γ region, within the IgG moiety, is designed to optimize recruitment of effectors, prolong the metabolic half-life ($t_{1/2}$),¹⁴ and minimize immunogenicity.¹¹ The first patient to be treated has undergone a partial remission of lymphoma and yielded data supporting the above contentions.

MATERIALS AND METHODS

Patient. B.R., a white woman, was seen in October 1983 at the age of 51 for a lump in the left axilla which on excision biopsy proved to be non-Hodgkin's lymphoma (centroblastic/centrocytic, nodular). No other lymphadenopathy was detected, but her spleen was palpable on inspiration. The blood showed hemoglobin (Hb) 13.3 g/dL, WBC count was $14.2 \times 10^9/L$, and platelet level was $225 \times 10^9/L$. The differential count was neutrophils 49%, lymphocytes 47%, and monocytes 4%; among the lymphocytes, small cleaved cells and centroblasts were prominent. Marrow aspirate revealed increased numbers of small cleaved lymphocytes, and paratrabecular infiltrates of lymphoma were seen on a trephine sample. Serum Ig levels were normal. Urinary Ig comprised κ 1.3 mg and λ 1.9 mg/24 hours; no clear evidence of a monoclonal component was seen on immunofixation of an isoelectric focusing pattern from concentrated urine. A lymphangiogram revealed enlarged nodes with abnormal patterns in paraaortic, parailiac, and inguinal regions. Studies of lymphocytic surface markers are summarized in Table 1. Blood lymphocytes stimulated with phorbol ester and pokeweed mitogen (PWM) showed Trisomy-12 on karyotyping. Leukapheresis in November 1983 yielded $\sim 10^{10}$ lymphocytes after separation over

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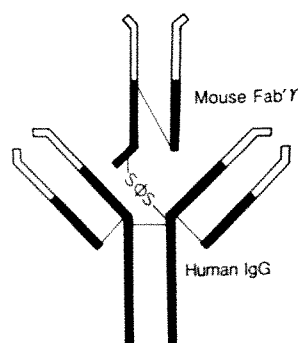


Fig 1. FabIgG prepared from the Fab'Y fragment of mouse monoclonal anti-idiotype antibody (anti-Id) and human normal IgG. Two thioether bonds link the two components through a phenylene ring. Attachment to Fab'Y is probably at a hinge 1/2-cystine as shown. Attachment to IgG has a high probability of being at one of the interchain 1/2-cystines, but is not known to show any preference among them. Residual interchain disulfide bonds are indicated by narrow lines.

Lymphoprep (Nyegaard, Oslo). These were used for studies in vitro and to derive tumor Ig for immunization.

The patient remained asymptomatic and was observed without treatment. In January 1985, examination revealed a splenic edge 4 cm below the costal margin and enlarged mobile lymph nodes as follows: left axilla 2×2 cm; left submandibular, two adjacent together 4.5×2 cm; left inguinal, two groups 4×1 and 2×2 cm; and right inguinal 1×1 cm. The leukemic overspill had not increased commensurately: WBC count was $11.2 \times 10^9/L$, lymphocytes 69%.

We decided to see whether the patient's tumor burden could usefully be reduced by antibody before resorting to conventional chemotherapy. Informed consent was obtained from the patient, and ethical clearance was granted by the East Dorset District Health Authority Research and Ethical Committee.

Marker studies. Blood lymphocytes and dispersed cells from the excised lymph node were prepared as described previously.¹⁵ The blood was drawn into preservative-free heparin. A portion of lymph node was minced and teased in Eagle's minimal essential medium (MEM) containing preservative-free heparin (20 U/mL), and rinsed through nylon gauze. Cells from both sources were then treated similarly. After separation over Lymphoprep, they were washed three times with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Before being stained, they were incubated for 60 minutes at 37 °C in BSA (2%)-MEM to remove

extrinsic Ig bound to Fc receptors. Sources of monoclonal antibodies were as follows: UCHT1, University College Hospital, London; RFA1, Royal Free Hospital, London; J5, Coulter Electronics, Luton, England; FMC4 and FMC7, SeraLab, Crawley Down, Sussex, England. Rabbit polyclonal anti-Ig reagents were visualized by direct immunofluorescence, UCHT1 and FMC4 by immunoperoxidase technique, and the other monoclonal antibodies by indirect immunofluorescence. Percentages of cells forming rosettes with sheep erythrocytes were determined as described previously.¹⁵

Ig for immunization. Idiotypic IgM λ as a source of immunogen was "rescued" for secretion in culture by fusing the patient's tumor cells with the mouse myeloma line 653. This technique yields a xenohybridoma that secretes Ig derived from the human tumor.¹⁶ For fusion, disaggregated cells from the patient and myeloma cells were mixed at a ratio of 2:1 in polyethylene glycol (PEG) 4000 (Merck-Schuchardt, Munich), washed in Dulbecco's MEM (DMEM) (GIBCO). Then, while still clumped from fusion, the cells were incubated at 37 °C for 2 to 4 hours in the DMEM containing 20% fetal calf serum (FCS); 2 mmol/L of L-glutamine; 1 mmol/L of sodium pyruvate, penicillin, and streptomycin both at 100 IU/mL; and HAT (10^{-4} mol/L of hypoxanthine, 4×10^{-7} mol/L of aminopterin, and 1.6×10^{-5} mol/L of thymidine). Finally, the cells were suspended and distributed into wells of a 96-well microculture plate (GIBCO, Paisley, Scotland), at $\sim 2 \times 10^5$ patient cells per well on a feeder layer of washed mouse blood cells. Colonies were screened by enzyme-linked immunosorbent assay (ELISA) for production of human IgM. Positive colonies were cloned at regular intervals by limiting dilution to guard against spontaneous loss of Ig chain production.

Monoclonal anti-Id. Xenohybridoma culture supernatant containing rescued idiotypic IgM was used as a source of immunogen for the immunization of Balb/c mice (Avonvale, Ashley Heath, England). The idiotypic 19S IgM was given in the form of purified immune complexes formed using mouse anti-human Fab μ serum by the method we described previously for the production of sheep polyclonal anti-Id serum.¹⁵ Each animal received complexes containing 10 to 50 μ g idiotypic IgM in 0.2 mL of complete Freund's adjuvant (CFA, Difco), distributed among four subcutaneous (SC) sites along the back. Two weeks later, animals were boosted by intravenous (IV) injection of a similar antigen dose in 0.2 mL of aqueous medium. Immune splenic lymphocytes were prepared 3 to 4 days later for fusion with the mouse myeloma line 653 by the method described for rescue of idiotypic IgM. Supernatants from the resulting hybridoma cells were assessed for reactivity with idiotypic IgM and normal human IgM by double-determinant ELISA.^{17,18} Those showing strong reactivity with the former but none with the latter were assessed further for reaction with the patient's tumor cells by indirect immunofluorescence, both with and without pooled normal human serum.¹⁸ (Reactivity not blocked by the normal serum can only be directed against highly private idiotypic determinants.⁸)

Suitable anti-Id-producing cells were cloned by limiting dilution and then expanded for large-scale antibody production in pristane-primed Balb/c $\times$ CBA F1 mice. Antibody IgG was prepared from ascitic fluid by sequential precipitation with 2 mol/L of $(NH_4)_2SO_4$ and chromatography on Trisacryl-M-diethylaminoethanol (DEAE) (LKB-Produkter, Bromma, Sweden). The DEAE column was equilibrated in 5 mmol/L of sodium phosphate, pH 8.0, and the IgG was eluted using a linear gradient of 5 to 50 mmol/L of sodium phosphate at constant pH. For all therapy and analytical procedures described in this article, two IgG1 antibodies were pooled: number B5G1 (5.5 g) and A4E2 (1.6 g). Both yielded good immunofluorescence with the patient's cells in the presence of normal human serum, and neither gave any reaction with normal blood lymphocytes or with neoplastic B lymphocytes from three other patients. By flow cytometry, with one antibody fluoresceinated and the other not, they

Table 1. Lymphocytic Surface Markers

Reagent	Marker	Percentages of Positive Cells	
		Nodal Biopsy (Dispersed Cells)	Blood Lymphocytes
Sheep RBCs	CD2	20	11
Monoclonal antibodies			
UCHT1	CD3	25	15
RFA1	CD5	ND	80
J5	CD10	ND	0
FMC4	HLA-DR(β)	ND	36
FMC7	CD22	ND	8
Polyclonal purified anti-Ig	μ chain	66	71
	δ chain	30	10
	κ chain	0	2
	λ chain	74	85

ND, not done.

revealed strong cross-inhibition. "Anti-Id IgG1" henceforth refers to this pool of two antibodies.

F(ab')₂ was prepared from the pooled IgG1 preparation by digestion with papain under standard conditions¹⁹: IgG1 20 mg/mL, papain 0.2 mg/mL in 0.1 mol/L of acetate buffer of pH 4.2, incubated at 37 °C for 18 hours, followed by chromatography on Sephacryl S300 (Pharmacia, Uppsala, Sweden) equilibrated in 0.1 mol/L of Tris HCl, pH 8.0.

Preparation of FabIgG. The preparation of FabIgG has been described in detail.¹¹ F(ab')₂ from antibody is reduced with 2-mercaptoethanol under the minimal conditions, giving a high yield of monomeric Fab'γ. Next, the liberated SH groups are allowed to react with a surplus of *o*-phenylenedimaleimide to give Fab'γ bearing a small number of active maleimide groups attached predominantly to its hinge region. The Fab'γ then reacts with human normal IgG that has previously been reduced with a limiting amount of dithiothreitol to give an average of <3.0 SH groups per molecule. The two reactants are mixed in a molar ratio of Fab'γ:IgG = 1:2 and incubated at 5 °C for 24 hours. The FabIgG is then separated from larger products and from residual reactants by recycling chromatography through Sephacryl S300 (Pharmacia) equilibrated with PBS. Typically, ~600 mg of FabIgG is collected from the column system in ~1,100 mL. Some surplus normal IgG contaminates the tail of the FabIgG zone, but this is regarded as of no consequence for therapeutic purposes. The yield of Fab'γ in the chimeric antibody was usually ~40% of that used in the synthesis.

Infusion of antibodies. The procedure for infusion of antibodies was essentially the same for both IgG and FabIgG. Any aggregated protein was removed from the preparations by passage through Sephacryl S300 and was equilibrated with sterile, pyrogen-free, PBS. For FabIgG, this corresponded with the purification procedure described previously. From the S300, the final antibody-containing effluent was led through a polymyxin column²⁰ to remove any pyrogens, and then through a 0.22-μm filter (Millipore, Harrow, England) directly into a sterile IV giving set. Aliquots from the giving set were tested for pyrogens by the limulus amoebocyte method (Pyrogen test, Mallinckrodt, Round Spinney, Northants, England) and always proved negative.

Twenty-four hours before the infusion was started, allopurinol 300 mg daily was started. At this time also, a serum sample was examined by micro-Ouchterlony technique for precipitins to mouse Ig, the method being capable of detecting antibody at <10 μg/mL. One hour before the infusion, a cutaneous prick test was performed to test for immediate hypersensitivity to the antibody.

Infusions, consisting of 200 mg of anti-Id IgG1 or 380 to 580 mg of anti-Id FabIgG, were given in a volume of 600 to 1,200 mL over 6 hours. The infusions of FabIgG were accompanied by up to 300 mg of human normal IgG, representing incompletely separated surplus reactant. A close watch was kept on temperature, pulse, and respiration.

Microassays. Our general procedure for ELISA has been described.¹⁷ For measurement of either FabIgG or mouse IgG in the patient's serum, the plate was coated with affinity-purified rabbit anti-mouse IgG at 5 μg/mL; this antibody had previously been absorbed with human Ig. The standard antigen was FabIgG or mouse IgG, corresponding to the unknown, used at starting concentrations of 200 and 20 ng/mL, respectively. Detection of bound antigen used the same rabbit anti-mouse IgG, conjugated to horseradish peroxidase and used at a dilution of 1:500.

To assay the patient's serum for the presence of idiotype-positive IgM, the ELISA plate was coated with monoclonal anti-Id using a solution at 5 μg/mL; the standard antigen was the supernatant of the IgM-secreting rescue hybridoma, and the detecting antibody was affinity-purified goat anti-human μ chain conjugated to horseradish peroxidase (1:1000) used with normal mouse serum (1:500).

Assessment of antibody in vitro. Tests for antibody-mediated complement lysis and for antibody-dependent cellular cytotoxicity (ADCC) were carried out by standard methods as previously described.²¹ Distribution of surface antigen-antibody complexes was visualized by indirect immunofluorescence,¹³ with flow cytometry carried out on the Becton Dickinson FACS III system.

RESULTS

Studies on tumor cells in vitro. Markers found on nodal and blood lymphocytes are summarized in Table 1. The surface Ig (IgMλ, with IgDλ just detectable on a minority of cells) was at a low level on all cells, being about equal to the average density observed in cases of chronic lymphocytic leukemia (CLL). About half the cells showed detectable class II histocompatibility antigen, whereas all stained for CD5, a pan-T marker that also appears on most cases of CLL and on some B cell lymphomas; in our experience, these findings are exceptional for this type of lymphoma.

A sample of blood lymphocytes prepared in February 1984 appeared from marker studies to consist of at least 80% tumor cells. When the cells were cultured for 6.5 hours in vitro, they secreted Ig as follows²²: κ chain 1500 and λ chain 6,400 dimeric molecules per cell per hour and IgM 130 monomeric molecules per cell per hour. This represented minimal IgM secretion by the tumor, and led to the expectation, confirmed later, that only a small barrier of idiotype Ig existed in the patient's plasma.

As we observed earlier⁵ with tumor cells having sparse surface Ig, no anti-Ig antibody tried would invoke complement lysis, using either human AB or rabbit serum as a source of complement. The antibodies tried included the monoclonal anti-Id, the FabIgG prepared from it, sheep polyclonal anti-Fdμ, and sheep polyclonal anti-λ. Neither would any of these antibodies invoke ADCC in the presence of human normal blood lymphocytes at an effector to target ratio of 50.

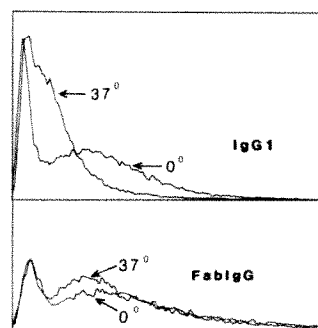


Fig 2. Modulation of blood lymphocytes in vitro by IgG1 and FabIgG anti-idiotype antibody (anti-Id) shown by cytofluorimetry of indirect immunofluorescence. x-axis, fluorescence (linear scale set at half-maximum gain); y-axis, cell number. Indicator antibodies: for IgG1, fluoresceinated rabbit anti-mouse IgG; for FabIgG, fluoresceinated rabbit anti-human Fcγ. Control (0 °C): cells (1.5 × 10⁷/mL) and antibody (IgG1 100 μg/mL, FabIgG 250 μg/mL) were incubated at 0 °C for 30 min; indicator antibody was then added, and the suspension was maintained chilled during examination. Test (37 °C): cells and antibody were incubated at 37 °C for 30 min; they were then chilled, and indicator antibody was added. FabIgG at 37 °C induced only a minimal change in profile. Peaks nearest the y-axis are antigen negative.

Modulation, in the sense of clearing of antigen-antibody complexes from the tumor cell surfaces, occurred sluggishly in vitro in the presence of monoclonal anti-Id at 37 °C: after 30 minutes, possibly 50% of the integrated fluorescence seen on flow cytofluorimetry had disappeared. Modulation by FabIgG over the same period was slight or undetectable (Fig 2), whereas modulation by its Fab γ component alone was never observed.

Infusion of anti-Id IgG1. An infusion of 200 mg over 6 hours was tolerated without any symptoms. Changes seen in the blood lymphocyte count are shown in Table 2. The lowest count recorded occurred in midinfusion; from this point, the count rose steadily to its former level over 1 week. The tumor masses did not change. This is very similar to what we have seen previously with infusions of polyclonal anti-Id^{3,5} and to what has been recorded for therapy with a variety of monoclonal antibodies.¹

Infusions of FabIgG. The clinical effects of each of four infusions were very similar. No symptoms were noted during the infusions. Beginning 6 hours after completion and continuing for 24 hours, the patient suffered nasal congestion, edema of the eyelids, mild pyrexia (37.5 °C), and mild abdominal pain. Four days after completion of infusion, lymph nodes in all areas were swollen, reaching about twice their previous size, remaining enlarged for 5 to 8 days, and then subsiding. There were no corresponding changes in splenic size.

Lymphocyte counts at midinfusion showed falls similar to that observed with IgG1 antibody (Table 2). The postinfusion nadir did not occur immediately after the infusion, however, but ~1 day later. From this point, the count again rose slowly, with the troughs more prolonged than after the IgG1 infusion.

A rise in C3c concentration in serum was evidence of complement activation. Thus, 7 hours after the second FabIgG infusion was started, crossed immunoelectrophoresis gave a strong peak for C3c. This was maximal at 24 hours and subsiding at 48 and 72 hours.

Following the third FabIgG infusion, with the associated nodal swelling and subsidence, a marked net diminution in the size of palpable tumor was obvious. Physical examination showed that the cervical masses had shrunk to $\leq 50\%$ of their initial volume and that the splenic edge had retreated to the costal margin. In the 11 weeks following the fourth FabIgG infusion, the tumor masses either remained stationary or subsided further, whereas the blood lymphocyte count dimin-

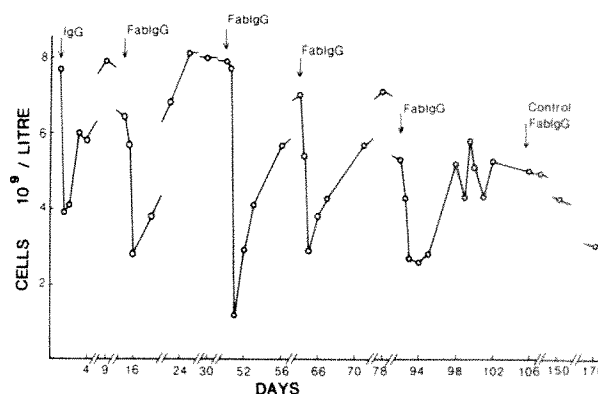


Fig 3. Serial lymphocyte counts related to antibody infusions. Day 1 is the day of IgG1 infusion (January 15, 1985).

ished to $3.1 \times 10^9/L$, ~40% of the pretreatment level (Fig 3).

In the ensuing 4 months, the patient remained well, with tumor masses unchanged and hemoglobin and granulocyte levels well maintained. The lymphocyte count, remarkably, dropped even more, to $2.4 \times 10^9/L$. Of these cells, >80% were idiotype-positive and only 7% were scored as T cells. On the tumor cells, the surface Ig appeared to be at about its original density, and reaction with the monoclonal anti-Id was undiminished. At no time during or after antibody treatment could antibody to mouse Ig be detected in the patient's plasma.

Plasma idiotype and antibody levels. Throughout the period of treatment, the plasma idiotypic IgM was at 0.9 to 1.4 $\mu g/mL$, except during the immediate postinfusion periods when, with free antibody in the plasma, the concentration fell to <0.3 $\mu g/mL$. These levels are much lower than those we have found in most B cell neoplasms.⁸

After all antibody infusions, both IgG1 and FabIgG, the antibody (assayed as mouse Fab γ) disappeared from the plasma with a $t_{1/2}$ of <24 hours. In the later FabIgG infusions, this was regarded with disappointment, as calculations had suggested that the antibody should have been sufficient to swamp both the extracellular and the cell surface idiotypic IgM. We believed it was important to confirm that the FabIgG was being consumed rapidly because of its antibody activity and not because of some unexpected metabolic fate such as scavenging by macrophages. The patient therefore consented to a control infusion

Table 2. Lymphocyte Counts Following Antibody Infusions

Antibody Preparation	Day of Infusion	Counts ($10^9/L$) at Times Related to Starting Infusions						
		-1 h	3 h*	7 h	1 day	2 days	3 days	6-8 days
IgG1								
200 mg	1	7.8	2.5	3.9	4.1	6.0	5.8	7.9
FabIgG								
480 mg	15	6.4	2.5	5.7	2.8		3.7	6.8
380 mg	50	7.9	3.5	7.7	1.2	2.9	4.1	5.6
450 mg	64	7.0	1.4	5.4	2.9	3.8	4.3	5.7
580 mg	92	5.3	2.2	4.3	2.7	2.6	2.8	5.1

*At midpoint of infusion.

of FabIgG in which the Fab'γ moiety had anti-Id activity against guinea pig L₂C cells and no activity against either her tumor cells or normal human lymphocytes; the IgG moiety was again normal human. Two weeks after the fourth therapeutic lot of FabIgG, the patient received a 6-hour infusion of 150 mg of the control material. There were no symptoms and no significant changes in lymphocyte counts. Plasma levels of mouse Fab'γ estimated for 1 week after the infusion revealed a $t_{1/2}$ > 10 days (Fig 4), comparable with the value of ~20 days expected for human normal IgG.²³

Qualitative studies of tumor cells. Reports on blood films from the hematology laboratory always indicated an increase in "smear" cells among the lymphocyte populations in immediate postinfusion periods: for example, these cells usually represented 10% to 15% of lymphocytes before an infusion but were reported at 50% 24 hours after the IgG1 antibody and at 43% 24 hours after the first lot of FabIgG antibody.

Observations of bound antibody and of modulation of surface antigen-antibody complexes were rendered difficult by the sparseness of surface Ig. Immediately after the IgG1 infusion immunofluorescence detected a diminution in the surface Ig; this was attributed to modulation. More surprisingly, flow cytofluorimetry revealed similar decreases in surface Ig after infusions of FabIgG. In Fig 5, the distributions of surface Ig on blood lymphocytes are shown at times related to the first FabIgG infusion. At 7 hours, 1 hour after the end of the infusion, all three anti-Ig antibodies used revealed diminished reactivity. At 24 hours, the profiles were essentially unchanged. At 3 days, the reactivities were still not completely restored, especially with anti-Id. Very similar appearances occurred after the ensuing three FabIgG infusions.

The diminution in detectable surface Ig may have been due to blocking of indicator antibodies by therapeutic antibody, to modulation, or to preferential removal by therapeutic antibody of cells with higher than average densities of surface Ig. We believe that modulation is probably the major cause, although patching and internalization could not be judged confidently by fluorescence microscopy. Residual therapeutic antibody could not have had a significant blocking role, as none could be detected on the cells by fluorescein-

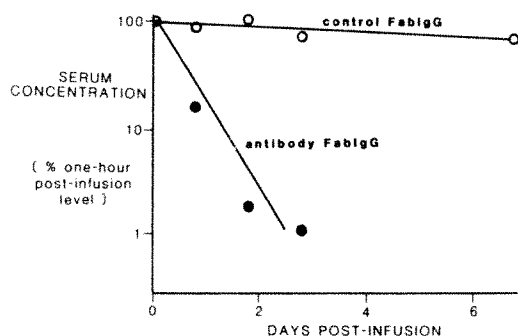


Fig 4. Rates of disappearance of antibody and control FabIgG from the patient's plasma. The antibody was the fourth infusion of anti-idiotype antibody (anti-Id) FabIgG. The first samples assayed were taken 1 hour after a 6-hour infusion.

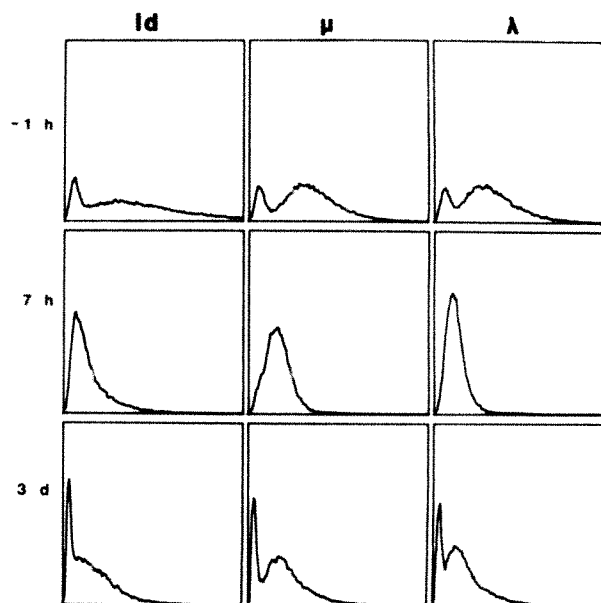


Fig 5. Surface Ig on blood lymphocytes before and after the first infusion of anti-idiotype antibody (anti-Id) FabIgG, demonstrated by flow cytofluorimetry. Axes: horizontal, degree of fluorescence; vertical, cell number. Idiotypic, μ and λ determinants were examined separately and are shown in separate columns. Idiotype was revealed by indirect immunofluorescence with anti-Id IgG1 as first antibody and fluoresceinated rabbit anti-mouse IgG as second antibody; μ and λ chains were revealed by direct fluorescence using affinity-purified rabbit antibodies. Time 0 is the beginning of the 6-hour infusion. Antigen-negative cells form a left-hand peak in the patterns at -1 hour and 3 days, but merge with the Ig-bearing peak in the 7-hour patterns.

ated anti-human Fcγ. Finally, the diminution in lymphocyte count at 7 hours, even if highly selective, does not seem sufficient in itself to have yielded so pronounced a change in surface Ig profiles. At no stage related to any of the infusions was the complement component C3 detected on blood lymphocytes by immunofluorescence.

Needle aspirates of cervical tumor masses were taken 14 days after the first FabIgG infusion as the masses were subsiding, and 2 days after the third FabIgG infusion before any infusion-related changes in size occurred. In the first aspirate, cytospin preparations revealed 74% tumor cells (λ -bearing) and 23% T cells (CD3-bearing). In the second aspirate, these figures were 69% and 21%, respectively; the tumor cells were predominantly large and atypical, many with one to three nucleoli. In neither preparation could the infused antibody be detected on cell surfaces.

DISCUSSION

No conclusions about the therapeutic efficacy of FabIgG are drawn from this case except that the derivative is at least capable under certain conditions of reducing tumor load. Non-Hodgkin's lymphoma is notorious for waxing and waning without obvious cause or following an apparently irrelevant event such as intercurrent infection.^{24,25} In the present case, there is no obvious reason for the reduced amount of tumor having apparently stabilized after termination of the

infusions, with indeed an actual further reduction in the number of circulating neoplastic cells. Clearly, one must bear in mind the possibility that antibody treatment of lymphoma may not only be cytotoxic, but may also trigger poorly understood immunoregulatory pathways.²⁶

Because of overall disappointing results in antibody therapy in lymphoma,^{1,5-7} some modification of the approach appears indicated. Apart from the mere addition of antibody to existing drug schedules, two main lines of development are being considered. One is the use of antibody as a vector to deliver a more lethal agent (radioisotope, tumoricidal drug, plant or bacterial toxin) to the cell. The other, adopted here, attempts to make better use of the cytotoxic effector mechanism normally recruited by antibody. The best known of these mechanisms are complement, phagocytes, and K cells. Unfortunately, we have no clear indications of their relative importance in dealing with antibody-coated lymphoma cells.⁸ We can assert, however, that a major advantage of relying on any of these mechanisms is that they contain an inbuilt safety factor in addition to the specificity of antibody: to activate any of the effectors, antibody must form a sufficient array on the cell surface, oriented with its Fc zones outward. Weak cross-reactions of the antibody with normal cells or the uptake of antigen-antibody complexes by bystander cells are unlikely to be harmful.

Univalent derivatives of antibody which retain intact Fc zones are superior to their bivalent parent antibodies in invoking killing of mammalian cell targets by natural effector mechanisms.^{9-11,27} Their superiority derives from their avoidance of rapid antigenic modulation, which can afford significant protection to a cell confronted simultaneously by bivalent antibody and an effector such as complement.^{13,28} The chimeric univalent antibodies FabFc¹⁰ and FabIgG (Fig 1) are developments of the univalent concept, presenting additional advantages from the possession of a human Fc γ zone. Such derivatives clearly are potentially varied and—especially if one includes molecular genetic and hybridoma technologies—might be constructed in a large variety of ways.

With the target cells of this study, neither FabIgG nor the parent IgG1 antibody appeared to activate complement or ADCC *in vitro*. We suspect that the failure of FabIgG is due to the sparseness of surface antigen.⁵ Against different target cells, chimeric univalent antibodies, including FabIgG, have clearly demonstrated their abilities to activate both the effector systems.^{11,29,30}

Despite their lack of cytotoxic activity *in vitro*, both IgG1 and FabIgG antibodies reduced the numbers of circulating neoplastic cells after infusion. If each drop in lymphocyte count reflects cell death, the effector mechanisms must be more useful *in vivo* than is suggested by performance *in vitro*. But could the drop be due more to temporary sequestration than to death of the cells? Attempts to answer this have been made in earlier studies^{31,32} by infusing antibody after indium-labeling of the blood lymphocytes. The results suggested cell death as the main mechanism because labeled cells disappearing on antibody infusion did not subsequently reappear in the blood in quantity. In the present work, the simple observation of a marked increase in smear cells

suggests a range of damage compatible with extensive cell death.

We are not aware of any previous report of tumor becoming swollen and tender in response to antibody therapy in the manner shown by the nodal masses in our patient. Because it occurred only with FabIgG infusions, and because the FabIgG but not the IgG1 antibody activates complement, it is tempting to regard this inflammation as complement-induced. The delay in onset for several days after each infusion is then difficult to explain, however. Examination of nodal aspirates did not help: in particular, granulocytes evidently were not recruited by complement peptides. Unfortunately, our patient was unwilling to undergo an excision biopsy that might have revealed more useful information. Garcia and colleagues³³ recently described the lymphoid histology in ten patients treated with monoclonal anti-Id, with evidence that clinical remission is more likely the greater the number of infiltrating T cells and the greater the incidence of CD4 (helper), CD25 (TAC), and Leu-7 (NK) positivity among them.

The mild symptoms suffered by the patient ~6 hours after each FabIgG infusion were possibly due to complement anaphylatoxins and/or to interleukin-1 from stimulated macrophages.

The modulation of surface Ig which appeared to be induced by FabIgG *in vivo* exceeded anything inducible by the same derivative *in vitro*. Evidence shows for the mouse Ia system that antibodies that do not modulate *in vitro* can do so *in vivo*.³⁴ Monocytes in the capacity of bystander cells have clearly promoted or even induced modulation of antibody on target cells,³⁵ raising the possibility that enhanced modulation *in vivo* in the present case was due to interaction of the bound FabIgG with FC γ -receptors on various cells.

Although the modulation by bivalent and univalent antibodies *in vivo* appeared similar, the time course cannot be followed with the same precision as is possible *in vitro*. The speed with which bivalent anti-Ig can induce modulation *in vitro* is sufficient to provide significant protection to a cell exposed simultaneously to antibody and complement.¹³ Neither we nor others observed such rapid modulation by univalent antibody.^{12,13} It therefore remains entirely possible that the modulation induced by univalent antibody *in vivo* is slower, and hence less protective, than that induced by bivalent antibody.

At the time of the fourth FabIgG infusion, the total idiotypic IgM harbored by the patient was estimated crudely at <50 mg based on a plasma level of 1 μ g/mL, an extravascular fluid load equal to the intravascular fluid load, a tumor burden <2 $\times 10^{12}$ cells, 20,000 molecules of monomer per cell surface, and an intracellular pool of the same size. The rate at which the infused antibody disappeared, in contrast to nonantibody FabIgG (Fig 4) indicates that our calculation was a gross underestimate. (Another explanation for the much briefer survival of the antibody FabIgG is that the patient herself was mounting an anti-Id response against the antibody. No increase in blocking activity was present in her plasma to support this suggestion.) Possibly, the large spleen contained unsuspected quantities of idiotypic Ig, although findings by Meeker and

co-workers⁶ on two similar patients subjected to splenectomy do not support this notion. It can be expected that saturation of antigenic sites would leave surplus antibody FabIgG with $t_{1/2}$ comparable to that of the control. With such a metabolic survival, the FabIgG would represent quite an economic means of using antibody to provide a prolonged attack on the tumor.

No anti-mouse Ig response has been observed in this patient. In animals, FabIgG containing homologous IgG but xenogeneic Fab elicits a much lower antibody response than does the corresponding xenogeneic IgG.¹¹ Therefore, there are grounds for hoping that avoidance of xenogeneic Fc γ will reduce the problem of anti-Ig responses by the patient

following therapy with monoclonal antibody. The idiotypic and mouse Fd γ determinants on FabIgG remain potential immunological targets. We shall continue to observe this patient closely. Our present intention is to reinstitute antibody treatment when the neoplasm appears to be expanding again.

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Plasminogen Activator Inhibitor: A Regulator of Ancrod-Induced Fibrin Deposition in Rabbits

By Chitra Krishnamurti, Charles F. Barr, M. Alycia Hassett, G. David Young, and Barbara M. Alving

Plasma levels of a fast-acting plasminogen activator inhibitor (PAI), which neutralizes both tissue plasminogen activator (t-PA) and urokinase, are markedly increased in endotoxin-treated rabbits. The ability of this inhibitor to prevent the fibrinolysis that occurs after a thrombogenic stimulus was investigated in a rabbit model. Normal and endotoxin-treated male New Zealand rabbits were infused with ancrod, an enzyme that causes noncrosslinked fibrin formation *in vivo*. Ancrod stimulated t-PA activity by 90% in normal rabbits and caused hypofibrinogenemia but did not increase PAI levels or induce fibrin deposition in target organs. Rabbits injected with endotoxin (10 µg/kg)

showed an increase in PAI from <1 to 32 U/mL 4 hours later. When ancrod was infused at this time, 90% of the rabbits developed renal fibrin thrombi. Fibrin deposition was recorded in 40% of the rabbits that received a lower dose of endotoxin (1.0 µg/kg) and had a PAI level of 14 U/mL at the time of ancrod infusion 4 hours later. Fibrin deposition did not occur in the endotoxin-treated rabbits that received normal saline. These data suggest that high levels of PAI inhibit fibrinolysis *in vivo*, thereby promoting fibrin clot deposition following a thrombogenic stimulus.

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TWO CRITICAL FACTORS in the regulation of fibrinolysis *in vivo* are the functional levels of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI), both of which are identical to those produced by endothelial cells.¹⁻³ PAI, a 50,000-mol-wt protein, has an apparent half-life ($t_{1/2}$) of 7 minutes when measured in the rabbit circulation⁴ and is the major inhibitor of t-PA and urokinase in both humans⁵ and rabbits.^{4,6} Elevated levels of PAI have been found in plasma from patients who have had venous thromboses,^{7,8} gram-negative sepsis,^{4,9} and many other conditions predisposing to fibrin clot formation.¹⁰

Colucci and co-workers⁴ demonstrated that infusion of endotoxin into rabbits increases PAI levels in a dose-dependent manner; they postulated that high levels of this inhibitor may have a physiologic role in the coagulopathy associated with endotoxemia. Although other investigators showed that infusion of soluble fibrin into endotoxin-treated rabbits results in renal microclot formation,¹¹ whether the apparent inhibition of fibrinolysis by endotoxin is mediated through its ability to increase levels of circulating PAI is not known.

The present study used endotoxin-treated rabbits to determine if elevated PAI levels could inhibit the fibrinolysis that occurs after infusion of ancrod, an enzyme that induces non-crosslinked fibrin clots by cleaving fibrinopeptides A, AP, and AY.¹²⁻¹⁴ Ancrod causes hypofibrinogenemia and elevated titers of fibrinogen-fibrin degradation products (FDP-fdp) when infused slowly into rabbits and humans.^{15,16}

This study demonstrates that ancrod does not cause fibrin deposition in normal rabbits, presumably because of the

fibrinolysis that occurs in response to its initial coagulant action. Ancrod can, however, induce fibrin deposition in animals that have elevated levels of PAI. The data indicate that PAI may promote fibrin formation by preventing the endogenous fibrinolysis that occurs after a thrombogenic stimulus.

MATERIALS AND METHODS

Materials. Purified two-chain human t-PA was purchased from American Diagnostica, Greenwich, CT. The specific activity was 100,000 U/mg, as determined with an international urokinase standard; the data in this article are expressed in terms of this standard. If this preparation of t-PA were calibrated against the International Reference Preparation for t-PA,¹⁷ the specific activity would be ~500,000 IU/mg.¹⁸ Urokinase (specific activity, 100,000 U/mg) was purchased from Abbott Laboratories, North Chicago. Human fibrinogen and Cohn fraction III paste were obtained from Cutter Laboratories, Berkeley, CA. Plasminogen was purified from the paste by affinity chromatography¹⁹ on lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden); the specific activity was 20 CTA U/mg. Human thrombin (H-2) and human plasmin (7.3 U/mg) were supplied by the Office of Biologics, Bethesda, MD. The synthetic substrate D-Val-Leu-Lys-pNA (S-2251) was purchased from Kabi Diagnostica, Stockholm. Ancrod, an enzyme derived from the Malayan pit viper, was the generous gift of Knoll Pharmaceutical, Whippany, NJ, and endotoxin (*Escherichia coli* 0111:B4) was purchased from Difco Laboratories, Detroit.

Animal experiments. Male New Zealand white rabbits weighing between 2.3 and 3.5 kg were received from a single supplier at least 1 week before use and were given standard laboratory food and water *ad libitum*. Endotoxin was infused into a marginal ear vein during 5 minutes in a total volume of 3 mL of normal saline at a dose of 1.0 or 10.0 µg/kg. Ancrod (2 U/kg) was administered by an infusion pump in a total volume of 20 mL of normal saline into a marginal ear vein during 1 hour. The vasopressin analogue 1-desamino-8-D-arginine vasopressin (DDAVP, Armour Pharmaceutical, Kankakee, IL) was infused into a marginal ear vein during 10 minutes at a dose of 0.4 µg/kg in 10 mL of normal saline. Blood samples (0.9 mL) were collected from the uninfused marginal ear vein into polypropylene tubes that contained 0.1 mL of 2% EDTA and immediately centrifuged for 1 minute at 22 °C in an Eppendorf centrifuge 3200 (Brinkman Instruments, Westbury, NY). The plasma was immediately separated from the RBCs, and aliquots were stored at -70 °C or acidified in preparation for measurement of plasminogen activator.

Assay of plasminogen activator activity. Assay for plasminogen activator activity was performed according to the method of Wiman

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and colleagues.²⁰ Plasma was acidified with an equal volume of 1.0 mol/L of sodium acetate pH 3.9 and incubated at 22 °C for 15 minutes. An aliquot of this mixture was diluted to a final plasma concentration of 0.5% in 0.05 mol/L of Tris, 0.1 mol/L NaCl, and 0.1% Triton X-100 pH 8.8 (NEN, Boston), and incubated with 1 mmol/L of S-2251 and 0.3 mg/mL of plasminogen in the presence of fibrin (70 µg/mL) for 6 hours at 37 °C in a microtiter plate. The change in absorbance was measured at 405 nm with a Titertek Multiscan Spectrophotometer (Flow Lab, McLean, VA).

Assay of PAI activity. The PAI assay was performed as described by Chmielewska and colleagues.²¹ Human t-PA (10 µL, 2U/mL final) was incubated for 10 minutes at 22 °C with 40 µL of plasma that was undiluted or diluted in 0.02 mol/L of sodium phosphate, 0.1 mol/L of NaCl pH 7.3. Residual t-PA activity was measured after acidification and dilution of the sample as described previously. One unit of inhibitor is defined as the amount that inactivates 1 U of t-PA during the 10-minute incubation period.

Assay of plasmin-inhibitor activity. Plasmin (final concentration 0.6 CTA U/mL) was incubated for 20 seconds at 30 °C with rabbit plasmas diluted 1/20 and 3/40 in 0.05 mol/L of Tris and 0.15 mol/L of NaCl pH 7.4. After 20 seconds, a 30-µL sample was added to 1 mL of 0.1 mmol/L of S-2251 in 0.05 mol/L of Tris pH 7.4, and the change in absorbance at 405 nm was monitored with a Cary recording spectrophotometer (Varian Associates, Instrument Division, Palo Alto, CA). The results were expressed as the percentage of activity of a standard of pooled human plasma.

Plasma plasminogen assay. Plasma was mixed with an equal volume of 1.0 mol/L of sodium acetate pH 3.9 and diluted to final concentrations of 2% and 1% with 0.05 mol/L of Tris, 0.1 mol/L of lysine, and 0.1% human albumin, pH 7.4. A 200-µL mixture containing 0.05% to 2.5% plasma, urokinase (2,500 U), S-2251 (0.2 mmol/L) in Tris-lysine buffer was then incubated in a microtiter plate for 30 minutes at 22 °C, and the change in absorbance was compared with that obtained with a standard curve prepared from human plasmin of known activity.

Fibrin plate zymography. Plasma samples were electrophoresed in sodium dodecyl sulfate (SDS) on an 8% polyacrylamide gel that was then washed for 30 minutes in 0.1 mol/L of Tris pH 8 containing 0.1% Triton X-100.²² The gel was overlaid with a fibrin monolayer containing plasminogen, incubated at 37 °C for 16 hours, and photographed.²²

Fibrinogen and FDP-fdp. The fibrinogen concentration was determined spectrophotometrically after plasma was clotted with a calcium-thrombin solution for 1 hour, and the clot was washed with normal saline followed by solubilization in alkaline urea.¹⁵ The FDP-fdp were measured in a tanned RBC hemagglutination immunoassay that was modified for use in rabbits.¹⁵

Data analysis. The results of experiments were expressed as the mean ± SEM. Statistical analysis was performed with a Student's *t* test on paired samples.

Table 1. Measurement of PA Activity in Rabbit Plasma

Rabbit No.	PA (U/mL)		
	A	B	C
I	0.41	0.42	0.09
II	0.66	0.66	0.42
III	0.55	0.51	0.23
IV	0.74	0.60	0.34
Mean ± SEM	0.59 ± 0.07	0.55 ± 0.05	0.27 ± 0.07

Plasma was acidified, diluted to 0.5%, and assayed immediately (A); acidified, diluted, frozen at -70 °C for 24 hours and then thawed and assayed (B); acidified, frozen at -70 °C for 24 hours and then diluted and assayed (C).

Histopathology. Necropsies were performed on animals that died during anecrod infusion or were killed by a 5-mL intravenous (IV) injection of sodium pentobarbital (V-Pento C, 65 mg/mL) 1 hour after completion of the infusion. Within 20 minutes of death, tissues from all animals were immersion-fixed in neutral buffered 10% formalin, processed, embedded in paraffin, and stained with hematoxylin and eosin. Sections of lung, liver, kidney, and spleen were also stained with phosphotungstic acid hematoxylin (PTAH)²³ and examined for the presence of fibrin microthrombi by a pathologist who was unaware of the experimental protocol for the individual animals.

RESULTS

Measurement and characterization of PA in rabbit plasma. Although freezing acidified human plasma has been reported not to affect the PA activity,²⁰ freezing and storage of rabbit plasma at -70 °C caused a significant decrease in PA activity as compared with that of unfrozen samples (Table 1). When acidified rabbit plasma was diluted to a final plasma concentration of 0.5% in Triton X-100 buffer and then stored at -70 °C, the PA remained unchanged from that measured in fresh plasma. Therefore, all plasmas were stored at -70 °C after acidification and dilution in Triton X-100 buffer. The apparent lability of rabbit PA could not be explained on the basis of qualitative or quantitative alterations in activators or inhibitors. Both species have similar levels of PAI and PA⁶; by fibrin zymography, the mol wt of PA in rabbit plasma is 68,000, similar to that of human t-PA.⁶ The PAI in rabbit plasma has the same mol wt as the PAI that has been characterized in human plasma.⁴

Effects of anecrod infusion in normal rabbits. When anecrod was infused into normal rabbits, fibrinogen levels decreased from 2.2 ± 0.1 to 0.3 ± 0.1 g/L and FDP-fdp increased from a baseline titer of 1/32 to 1/4098 at 1.5 hours after initiation of the infusion. These alterations were associated with a significant increase in PA activity of 91.3% ± 16.1% (*P* < .01, Fig 1). Six hours later, the PA levels had returned to baseline values, and the fibrinogen levels were still depressed (0.4 g/L). When anecrod was infused at this time into these hypofibrinogenemic rabbits, PA activity was again increased by 40.7% ± 14.6% above the 6-hour value

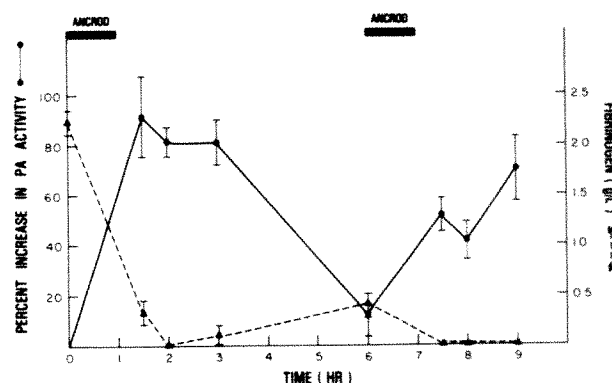


Fig 1. Effect of anecrod on plasminogen activator (PA) activity in hypofibrinogenemic rabbits. Anecrod (2 U/kg) was infused at 0 and 6 hours. PA activity (—) and fibrinogen levels (---) were determined at the indicated time points. Data are the mean ± SEM of four rabbits.



Fig 2. Fibrin zymogram of paired rabbit plasmas obtained before and 1.5 hours after infusion of ancrod or saline. Samples were electrophoresed in sodium dodecyl sulfate (SDS) on an 8% polyacrylamide gel. Lanes 1,3, pre-ancrod samples, lanes 2,4, post-ancrod; lanes 5,7, pre-saline; lanes 6,8, post-saline; lane 9, human-melanoma tissue plasminogen activator (t-PA); lane 10, human urokinase.

($P < .05$) and fibrinogen levels became undetectable. The PA values remained elevated for another 2 hours after the second ancrod infusion. By fibrin plate zymography, the PA before and after ancrod was 68,000 mol wt (Fig 2), suggesting that the increase in PA after ancrod was t-PA. Ancrod had no effect on PAI levels, which were <1 U/mL when measured before and at 1.5 hours after infusion. Infusions of saline into six control rabbits caused no increase in PA, PAI, or change in fibrinogen levels (data not shown).

To determine if ancrod could stimulate release of PA in the absence of any detectable fibrinogen, a group of animals was rendered afibrinogenemic by infusing ancrod at 0 to 1 hour and again at 2 to 3 hours (Fig 3). At 6 hours, these afibrinogenemic rabbits received either a third infusion of ancrod or DDAVP, which is known to stimulate PA in normal rabbits by 40%.⁶ Ancrod caused no increase in PA when infused at this time; however, PA was significantly increased by 26% ($P < .05$) in the five rabbits that had received DDAVP. These data indicate that ancrod stimulates PA release only when infused into rabbits that have circulating fibrinogen, suggesting that clot formation induced by ancrod is the stimulus for PA release. The ability of the afibrinogenemic rabbits to release PA when infused with DDAVP indicates that the PA stores had not been completely depleted by two previous infusions of ancrod.

Ancrod-induced fibrin deposition in endotoxin-treated rabbits. Infusion of endotoxin into rabbits caused a dose-dependent increase in both PA and PAI (Fig 4A and B). One group of rabbits received endotoxin at a dose of $1 \mu\text{g/kg}$, followed by an infusion of normal saline or ancrod 4 hours later (Fig 4A). Approximately 90 minutes after endotoxin infusion, PA was maximally increased by $41.2\% \pm 10.5\%$ above baseline values ($P < .01$), returning to normal values by 4 hours. PAI levels increased from <1 U/mL to maximum values of 14.3 ± 2.3 U/mL at 4 hours. Infusion of ancrod at this time did not cause an increase in PA, although fibrinogen levels decreased from 1.7 ± 0.3 to 0.1 ± 0.1 g/L. At necropsy, 40% of the rabbits had evidence of renal thrombi (Table 2). One of these animals also had pulmonary thrombi. None of the endotoxin-treated animals that received saline instead of ancrod showed any evidence of fibrin deposition.

A second group of rabbits was infused with endotoxin at a dose of $10 \mu\text{g/kg}$, followed by ancrod or normal saline 4 hours later. After infusion of endotoxin, PA was maximal at

30 minutes and declined at 90 minutes as PAI levels rapidly increased (Fig 4B). By 4 hours, PAI had increased from <1 to 32 U/mL; plasminogen levels had decreased from 5.9 to 4.2 U/mL, and α_2 -antiplasmin was decreased by 36%. Thus, the most striking change in components of the fibrinolytic system 4 hours after this dose of endotoxin was the marked increase in PAI. Infusion of ancrod at this time caused glomerular fibrin deposition in 90% of the animals (Table 2). One of these animals also had pulmonary thrombi, and one demonstrated pulmonary and splenic thrombi in addition to the fibrin deposition in the glomeruli (Fig 5). The endotoxin-treated animals that received saline had no demonstrable fibrin deposition.

These data suggested that endotoxin, by increasing PAI levels, promoted fibrin deposition in the ancrod-treated rabbits. The following experiment was done to determine if ancrod induced clot deposition when infused into endotoxin-treated rabbits that had PAI levels <1 U/mL at the time of initiation of infusion. Ancrod was infused into rabbits 30 minutes after they had received endotoxin ($10 \mu\text{g/kg}$). PAI levels increased from <1 to ~ 16 U/mL during the ancrod infusion and were 24 U/mL 1 hour later, at the time of necropsy. Fibrin deposition was detected in 50% of these rabbits (Table 2).

All rabbits infused with ancrod alone or with endotoxin at a dose of $1.0 \mu\text{g/kg}$ followed by ancrod survived the infusion.

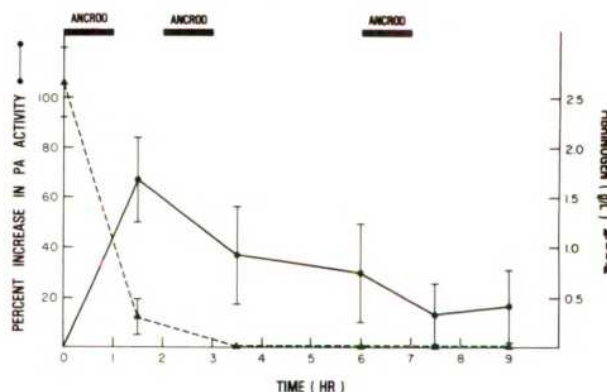
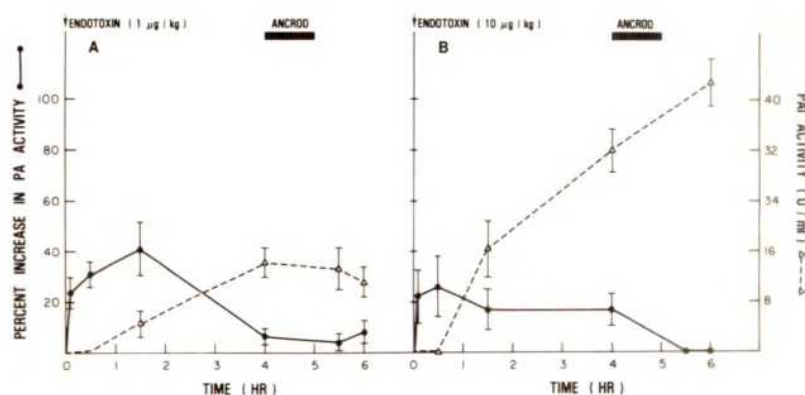


Fig 3. Fibrinolytic response in rabbits after multiple infusions of ancrod. Plasminogen activator (PA) activity (—) and fibrinogen levels (---) were measured at the indicated time intervals after ancrod infusions at 0, 2 and 6 hours. Results are the mean $\pm$ SEM of five rabbits.

Fig 4. Effect of ancrod on endotoxin-treated rabbits. Endotoxin was administered at doses of 1 $\mu\text{g/kg}$ (A, 12 rabbits) or 10 $\mu\text{g/kg}$ (B, 13 rabbits), and ancrod was infused 4 hours later. The plasminogen activator (PA) (—) and PA inhibitor (PAI) (---) activities (mean $\pm$ SEM) are shown.



Animals that received endotoxin at the higher dose of 10 $\mu\text{g/kg}$ followed by ancrod at 30 minutes or 4 hours had mortalities of 50% and 40%, respectively. The mortality was 14% for those animals that received only saline 4 hours after this dose of endotoxin. Mortality could not be correlated with the presence of fibrin deposition.

DISCUSSION

The present study demonstrates that when a thrombogenic stimulus such as ancrod is infused slowly into normal rabbits, PA levels increase as a result of the coagulant action of ancrod on fibrinogen. PAI levels are not increased and fibrin deposition does not occur after ancrod infusion alone. However, rabbits that have a high level of PAI at the initiation of ancrod administration or rabbits that are undergoing rapid increases in PAI during the infusion may develop fibrin

deposition, predominantly in the glomeruli. It is unlikely that alterations in other components of the fibrinolytic system are sufficient to be implicated in the thrombus formation that occurred in endotoxin-treated rabbits that received ancrod. Although plasminogen levels decreased after endotoxin infusion, α_2 -antiplasmin levels were lower as well.

The infusion of endotoxin also causes leukopenia, generation of cellular and humoral procoagulant activity, and activation of the complement system.²⁴ These alterations, however, do not appear to be significant factors in the induction of fibrin formation described in the present study. In their work with rabbits deficient in the sixth component of complement, Muller-Berghaus and Lohmann demonstrated that the complement system does not have a role in the induction of renal microclot formation in animals that receive a continuous infusion of endotoxin.²⁵ Muller-Berghaus and coworkers also reported that rabbits injected simultaneously with ancrod and endotoxin developed glomerular microclots that could not be prevented by heparin²⁶ or by first rendering the animals neutropenic with nitrogen mustard.²⁷ They concluded that the endotoxin-mediated generation of

Table 2. Summary of Histologic Studies

Study/Treatment	Fibrin Thrombi		PAI Levels (U/mL)* (Mean $\pm$ SEM)
	(%)	(No. of Animals)	
Experimental			
Endotoxin (1 $\mu\text{g/kg}$), ancrod (4 h)†	40%	(2/5)†	11.7 $\pm$ 3.1
Endotoxin (10 $\mu\text{g/kg}$), ancrod (4 h)	90%	(9/10)	33.3 $\pm$ 3.4
Endotoxin (10 $\mu\text{g/kg}$), ancrod (30 min)	50%	(3/6)	0.2 $\pm$ 0.1 ↓ 24.7 $\pm$ 3.4
Controls			
Endotoxin (1 $\mu\text{g/kg}$), saline (4 h)	0%	(0/4)	20.4 $\pm$ 6.0
Endotoxin (10 $\mu\text{g/kg}$), saline (4 h)	0%	(0/7)	33.3 $\pm$ 2.9
Saline, ancrod (4 h)	0%	(0/4)	2.0 $\pm$ 1.6

*Plasminogen activator inhibitor (PAI) levels at time of second infusion (ancrod or saline). For rabbits that received ancrod 30 minutes after endotoxin, PAI levels had not yet reached a plateau and increased from 0.2 to 24.7 U/mL at time of necropsy.

†Time of second infusion (ancrod or saline). After the administration of endotoxin, ancrod or saline was infused into the animals. Survivors were killed 1 hour postinfusion. Lung, liver, spleen, and kidneys were fixed, stained with phosphotungstic acid hematoxylin (PTAH), and examined for the presence of fibrin microthrombi.

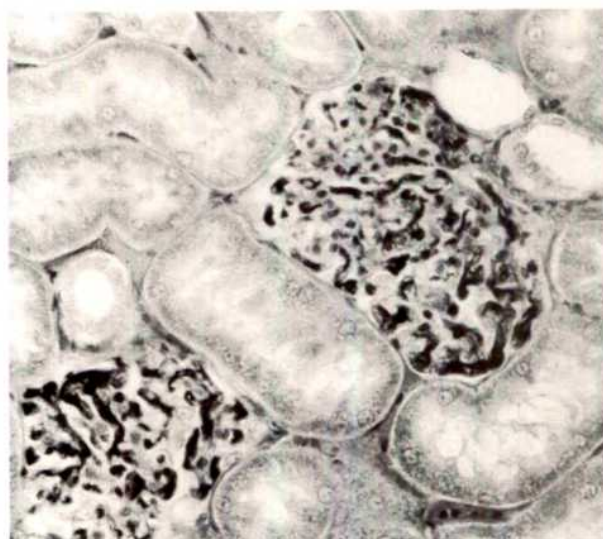


Fig 5. Histopathologic evidence of fibrin microthrombi (dark, fibrillar areas) in the glomeruli of a rabbit infused with endotoxin (10 $\mu\text{g/kg}$) and then ancrod 4 hours later. The animal was killed 1 hour after completion of ancrod infusion.

microclots in anecrod-infused rabbits did not involve neutrophils or the generation of procoagulant activity by endotoxin.

Additional indirect evidence for the role of PAI in inhibiting fibrinolysis is suggested by the studies of Szczepanski and Lucer,²⁸ who reported that the infusion of a single dose of endotoxin into rabbits caused an increase in the fibrinolytic activity of the plasma euglobulin fraction within the first hour, followed by a decrease in the plasminogen activator activity of kidney tissue at ~6 hours. Bergstein and Michael²⁹ found that fibrinolytic activity was present in the renal cortices of rabbits killed 30 minutes after a single dose of endotoxin but was undetectable in animals killed from 1 to 2 hours later. The activity reappeared at 24 hours, thus following a time course consistent with induction of PAI. The loss of fibrinolytic activity after infusion of endotoxin alone was not associated with fibrin deposition.

The vulnerability of the kidneys of the endotoxin-treated rabbits to fibrin deposition after anecrod may reflect the low level of PA activity relative to the levels of PAI within this particular organ. In the present study, only 3 of 14 rabbits that developed renal fibrin deposition had pulmonary thrombi as well. One explanation for these findings is that

pulmonary tissue can have an inhibitory effect on the function of PAI, as was recently demonstrated by Colucci and co-workers.¹⁸ These investigators showed that addition of rabbit lung slices to a whole blood clot markedly enhanced its lysis when it was incubated in the presence of PAI inhibitor-rich plasma *in vitro*.

The highest levels of PAI appear to be induced by endotoxin in humans^{4,9} as well as in rabbits. However, the levels of PAI required to prevent fibrinolysis are not known for specific clinical settings. The levels may depend on the type of thrombogenic stimulus that is acting in a given situation. For example, anecrod induces non-crosslinked fibrin formation which undergoes lysis relatively easily. The generation of small amounts of thrombin which causes activation of coagulation factor XIII with crosslinking of α_2 -antiplasmin to the clot³⁰ may be much more thrombogenic and require larger concentrations of activator for lysis.

Elevated PAI levels appear to be a significant factor for clot formation when a thrombogenic stimulus such as anecrod is administered. Monitoring levels of this inhibitor in patients, especially those in sepsis, may allow prevention of renal fibrin deposition, which is not uncommon in this clinical condition.³¹

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Culture From Human Bone Marrow of Blast Progenitor Cells With an Extensive Proliferative Capacity

By Scott D. Rowley, Saul J. Sharkis, Catherine Hattenburg, and Lyle L. Sensenbrenner

The investigation of human hematopoiesis is limited by the lack of an *in vitro* assay for the most primitive hematopoietic stem cell. In this report, we describe the culture from normal human bone marrow of unique colonies of morphologically immature cells with scanty, agranular, cytoplasm and a primitive nucleus with nucleoli. These "blast" cells demonstrate a significant ability for the generation of secondary colonies of multiple lineages, including additional blast cell colonies. These colonies are

detected at various times during the culture period of up to 28 days. Neither the time of appearance in primary culture nor any feature of the morphological appearance of the blast cells is correlated with replating ability or the differentiation pathway followed. The progenitor cell giving rise to these colonies may represent the earliest pluripotent hematopoietic stem cell yet grown in culture.

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THE CURRENT general model of hematopoiesis assumes that mature blood cells arise from pluripotent hematopoietic stem cells capable of both self-renewal and differentiation.¹ Increasing maturation of the progenitor cell results in the loss of its self-renewal capacity and, concomitantly, a restriction in differentiation to a particular cell lineage. The clonal growth *in vitro* of hematopoietic cells supports this theory and facilitates the investigation of hematopoietic function. The cells of colonies arising in cultures of committed hematopoietic progenitors lack the primary features of stem cells, however, ie, an extensive ability for self-renewal and the ability to give rise to progeny of multiple lineages when replated. The most primitive progenitor cell described in cultures of human bone marrow so far, the CFU-GEMM,^{2,3} demonstrates only a limited capacity for self-renewal.⁴⁻⁶

Recently, murine progenitor cells capable of extensive replating and self-renewal were reported by several investigators.⁷⁻⁹ The cells of these colonies are morphologically immature ("blast cells") and, when replated, may give rise to secondary colonies of various lineages including additional blast cell colonies. This particular progenitor cell in one assay system demonstrates a "variable G₀ phase," with colonies appearing apparently randomly throughout the culture period.¹⁰ The work of Nakahata and colleagues with this primitive murine progenitor cell supports a stochastic model for hematopoietic stem cell differentiation.¹¹ Cultures of human umbilical cord blood give rise to similar colonies,^{6,12} although secondary colony formation when the blast cells are replated⁶ is much poorer than that reported for the murine cultures.^{7,11} To date, a similar cell has not been reported in cultures of

normal human bone marrow. In this report, we describe the growth from human bone marrow of primitive pluripotent cells that also have an extensive capability for the generation of secondary colonies. These cells may represent the earliest human hematopoietic stem cell grown *in vitro*.

MATERIALS AND METHODS

Bone marrow cells were aspirated from the posterior iliac crests of healthy human donors after informed consent was obtained. The collection of normal human bone marrow specimens is approved by the Joint Committee on Clinical Investigation of The Johns Hopkins University School of Medicine and The Johns Hopkins Hospital.

Cell culture. The assay for human blast cell colonies is modified from the technique previously described by Nakahata and Ogawa.⁶ Specifically, light density (sp gr < 1.077) mononuclear bone marrow cells obtained from Ficoll-Hypaque density-gradient centrifugation were cultured in α -MEM (GIBCO, Grand Island, NY), supplemented with 1.32% methylcellulose (1500 CP, Fisher), 30% fetal bovine serum (FBS, Hyclone, Logan, UT), 5×10^{-4} mol/L 2-mercaptoethanol, 5% conditioned medium (phytohemagglutinin-leukocyte-conditioned medium, PHA-LCM or tumor cell CM; described later), 1% bovine serum albumin (Sigma, St Louis), 10^{-6} mol/L of methylprednisolone sodium succinate ester, and 1 U/mL of recombinant human erythropoietin (Amgen, Thousand Oaks, CA). Quadruplicate 1-mL cultures of 50,000 bone marrow cells per culture dish (Lux, Miles Laboratories, Naperville, IL) were maintained in a 5% CO₂ humidified atmosphere at 37 °C for up to 29 days. Colonies derived from BFU-E, CFU-GM, and CFU-GEMM progenitor cells were scored on an inverted microscope after 14 to 16 days of culture. The dishes were returned to the incubator, and blast cell colonies (>20 cells) were scored after 26 to 29 days of culture (or as otherwise noted). All blast cell colonies were plucked from the culture dishes with a 10- μ L Eppendorf micropipette and resuspended in 100 μ L of α -MEM supplemented with 10% FBS. For replating experiments, one-half of the colony was immediately replated. The remaining cells were centrifuged onto glass slides for cytochemical staining (Wright's, specific and nonspecific esterases) and morphological examination.

Replating experiments. All colonies for replating were plucked from the primary culture as described previously after an estimation of colony size was made by *in situ* cell enumeration. One-half of the colony was immediately replated in the same conditions used for the primary cultures. Secondary colonies were scored after 14 to 16 days of culture and again after 26 to 29 days of culture. Clusters and colonies that appeared for only a few days early after replating (transient colonies) were not considered.

Conditioned medium. PHA-LCM was obtained from light-density (sp gr < 1.077) peripheral blood leukocytes from healthy human donors. The cells were suspended at 10^6 cells/mL in α -MEM supplemented with 20% FBS and 1% vol/vol of PHA (Wellcome

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Laboratories, Beckenham, England). The flasks were maintained at 37 °C and 5% CO₂ for seven days. Cells and cellular debris were removed by centrifugation (400 g), and the supernatants were filtered (0.22 µm) and frozen in aliquots for use.

The CM¹³ obtained from culture of a hairy cell leukemia cell line (Mo-CM) was a gift of Dr David Golde (University of California at Los Angeles) and contained 5% FBS. The 5637-CM was derived from culture of a bladder tumor cell line.¹⁴

Nomenclature. We adopted the term blast cell for the cell giving rise to blast cell colonies in this assay.

Statistical analysis. Statistical analysis of the differences between numbers of colonies observed in cultures was performed using Student's two-tailed *t* test.

RESULTS

The blast cell colonies were loose homogeneous clusters of moderate-sized, translucent, round, agranular, and nonpigmented cells without evidence of myeloid or erythroid differentiation (Fig 1A); they appeared throughout the culture period (described later). This contrasted with the obvious red pigmentation of erythroid cells and the clearly mature cells with irregular outline, granular cytoplasm, and mature nucleus of BFU-E-derived and CFU-GM-derived colonies, respectively. Especially after 21 to 28 days of culture, the immature blast cell-containing colonies were easily distinguished from the more mature progenitor cell colonies, which

almost uniformly showed extensive degeneration. Also unlike colonies derived from the more mature progenitor cells, which on morphological examination frequently reveal cells comprising a wide range of maturation, the blast cell colonies consisted solely of blast cells with uniform morphological appearance (Fig 1B) except as noted below. Mitotic figures were commonly observed in cytospin preparations of blast cell colonies. The blast cells did not demonstrate either specific or nonspecific esterase staining. The blast cell colonies usually contained 200 to 500 cells, although colonies as small as 20 cells and as large as ~1,000 cells without maturation were observed. Frequently, the larger colonies contained a mixture of blast cells and foci of maturing cells. (These colonies were excluded from analysis since replates of these colonies invariably gave rise to very limited numbers of secondary colonies; CFU-GEMM replating efficiency is described below.)

The tumor cell CM supported increased blast cell colony growth as compared with the PHA-LCM (Table 1). This increase was approximately twofold to threefold and is statistically different ($P < .05$). Only a limited number of blast cell colonies were observed after 28 days of culture. We observed a mean ($\pm$ SEM, $n = 3$) of 3.7 ± 0.5 blast colonies in each milliliter of culture on day 14 of culture as compared with 0.9 ± 0.3 and 0.7 ± 0.2 on days 21 and 28 in a series of

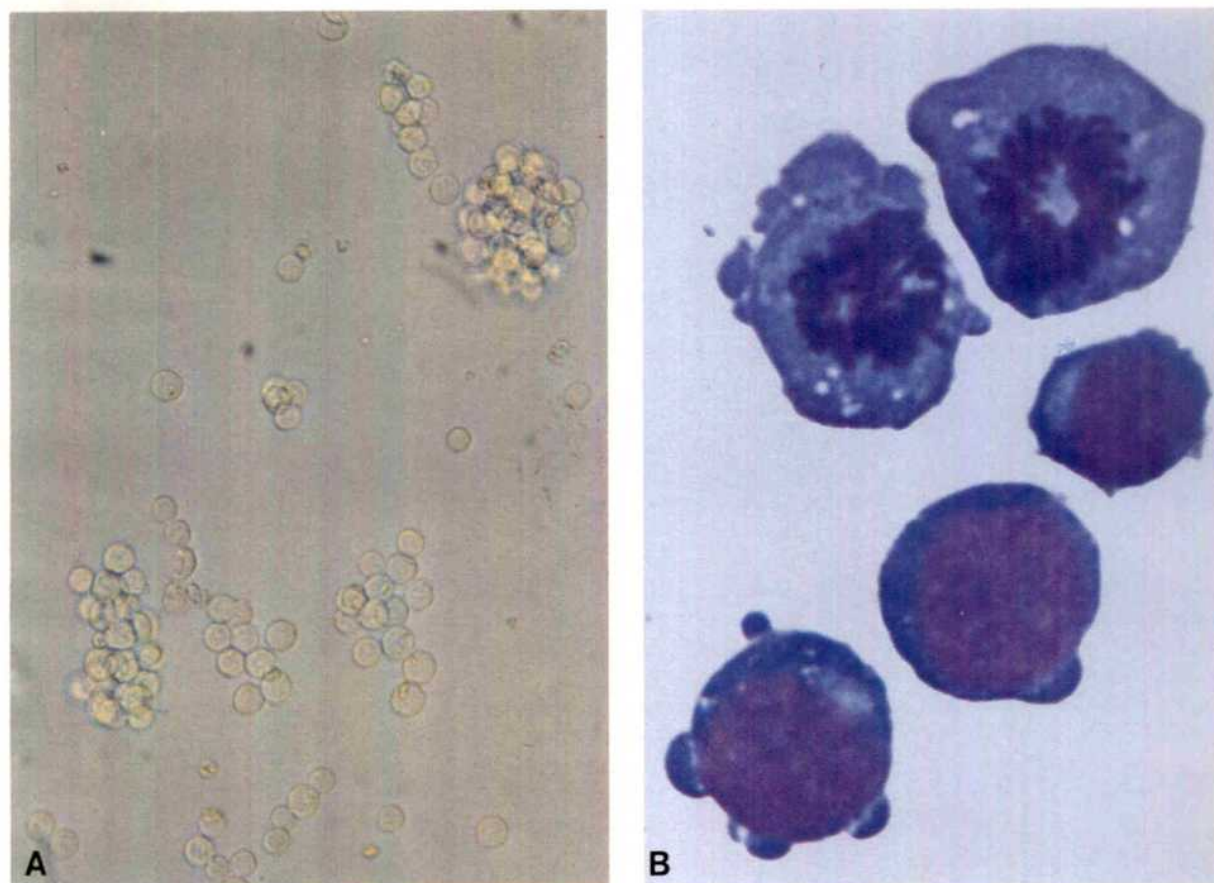


Fig 1. Photomicrographs of (A) blast cell colony in situ, magnification $\times 400$, and (B) cytospin preparation of blast cells stained with Wright's stain, magnification $\times 1,300$.

Table 1. Primary Colony Growth From Primitive and Committed Progenitor Cells

Conditioned Medium†	Observed Colonies*			
	BFU-E	CFU-GM	CFU-GEMM	Blast Cell
PHA-LCM	68.9 ± 12.4	78.2 ± 7.5	5.6 ± 1.2	0.4 ± 0.1
MO-CM	67.0 ± 8.6	66.7 ± 7.5	5.3 ± 0.7	1.2 ± 0.3
5637-CM	44.7 ± 9.7	56.0 ± 7.2	4.1 ± 0.9	0.8 ± 0.2

PHA-LCM, phytohemagglutinin-leukocyte-conditioned medium; Mo-CM, CM derived from a hairy cell leukemia line; 5637-CM, CM derived from culture of a human bladder tumor cell line.

*Colonies scored per 5×10^4 light-density bone marrow cells plated. Culture periods are described in the Materials and Methods section.

†Experiments for PHA-LCM and Mo-CM ($n = 9$); experiments for 5637-CM ($n = 8$).

experiments in which different sets of four plates were scored at each time point (difference in growth of blast cell colonies at day 14 and day 21 or day 28 was significant, $P < .05$). We did not score for blast cell colonies earlier than 14 days of culture to avoid confusion with the immature colonies of other lineages. Occasionally, small numbers of fully differentiated colonies without the usual extensive degeneration were observed after 28 days of culture. We presume that these colonies differentiated from blast cells present earlier in the culture period. Most (50% to 100%, mean 79%) of the blast cell colonies in this series contained progenitor cells that formed secondary colonies, and the replating efficiency was similar for the three time points. Blast cell colonies that did not replate (excluding colonies containing mature cells as described above) could not be distinguished on the basis of colony size or cytochemical staining of the individual cells from colonies with self-renewal capacity.

Blast cells dispersed into secondary cultures generated moderate numbers of secondary colonies of various lineages (Table 2), with some secondary blast cell colonies of 70 to 130 cells observed after an additional 28 days of culture. These secondary blast cell colonies have given rise to tertiary colonies (erythroid, granulocytic, not shown), but no tertiary blast cell colonies have yet been found. Mature granulocytic colonies replated after 14 or 28 days of primary culture and mature erythroid colonies replated after 14 days of culture did not give rise to secondary cultures. CFU-GEMM-derived colonies ($n = 10$) replated after 14 days of primary culture demonstrated some limited proliferative capacity. Three of these ten colonies, which contained an average of $2.8 \pm 0.5 \times 10^4$ cells, gave rise to 16, 7, and 16 granulocytic colonies, respectively. These replates had no secondary BFU-E, CFU-GEMM, or blast cell colonies.

The maturation pattern (Table 2) of colonies in the secondary cultures could not be predicted by the primary colony size or by cytochemical staining characteristics of cytospin preparations of the blast cells. We observed individual primary colonies giving rise to secondary colonies of erythroid or granulocytic lineages only, mixtures thereof, or some pluripotent progenitor cell colonies (CFU-GEMM or blast cell). Usually after 14 days of culture we did observe some primary colonies composed of cells similar to blast cells except appearing more opaque in situ. These cells contained early cytoplasmic granule formation with Wright's staining

and invariably gave rise to granulocytic colonies in secondary plating. Of 10 of these colonies from 5 bone marrow samples, 5 (50%) replated with a mean replating efficiency (number of secondary colonies grown per number of cells seeded) of 16.7%. These "late blast" colonies may have been derived from the more immature blast cell colonies, and may represent colonies that have become lineage restricted.

DISCUSSION

This report documents the presence in human bone marrow of hematopoietic progenitor cells that have a significant replating capacity suggestive of high proliferative potential and can be cultured in vitro. The colonies observed differ from those produced by the more mature progenitor cells by their variable time of appearance in culture (also seen in murine cultures of "S cells"¹⁰), in the uniform morphological appearance of the cells within and between blast cell colonies and, most importantly, in the ability to generate secondary colonies of multiple lineages, including additional pluripotent stem cell-derived colonies upon replating. We believe that these cells are functionally similar to those isolated from human umbilical cord blood^{6,13} and murine bone marrow and spleen.⁷⁻⁵ These cells may also be comparable to the human bone marrow progenitor cells giving rise to immature colonies in 5-day cultures on previously established adherent cell layers,¹⁵ although the differences in culture conditions make comparison difficult.

The tumor cell CM (Mo-CM or 5637-CM) resulted in increased growth of the blast cell colonies as compared with cultures of bone marrow with PHA-LCM. We did not determine if this increased growth relates to increased concentrations of particular growth factors contained within these media or the presence of an inhibitor(s) contained within the PHA-LCM. Additional evaluation of growth factor requirements for blast cell colony growth is under study. The observation by Keller and colleagues⁸ that initial co-culture of murine bone marrow cells with lymphocytes increased not only primary blast cell growth but also the secondary replating efficiency of the blast cells suggests that a specific growth factor (ie, lymphokine) may be important for optimal growth of the primitive stem cells. We did observe the growth of blast cell colonies from T cell-depleted cord blood,¹² which suggests the lymphocyte presence may not be required in this system, although lymphocytes may enhance the growth of blast cell colonies as shown for other progenitor cells.^{16,17}

We observed a marked heterogeneity of both the replating efficiency and lineage commitment in the secondary replates that could not be predicted by primary colony size or any other determined feature of morphological appearance. Mostly, we observed secondary replates containing only mature progenitor cell colonies (BFU-E, CFU-GM), although occasional CFU-GEMM and blast cell colonies were found. This definite tendency toward differentiation of the blast cells was also noted in human cord blood blast cell replates⁶; murine blast cells in one report retained significant self-renewal capacity,⁷ although blast cells from a different assay system have not been reported to generate any second-

Table 2. Replating Potentials of Blast Cell Colonies

	Colony No.*	Culture (day)†	No. of Cells Replated	BFU-E	CFU-GM	CFU-GEMM	Blast Cell
Four colony types in replates	1	28	75	15	103	3	3
	2	28	70	45	117	6	3
	3	14	75	8	11	3	1
Three colony types in replates	4	28	40	84	91	16	0
	5	28	200	14	124	1	0
	6	28	120	52	98	9	0
	7	28	200	20	109	4	0
	8	28	50	13	105	2	0
	9	26	116	19	5	1	0
	10	26	116	3	26	1	0
	11	14	40	19	3	0	1
	12	14	38	2	2	4	0
	13	21	125	2	7	5	0
	14	28	38	10	15	1	0
	15	28	100	41	15	2	0
	16	14	200	31	46	2	0
	17	14	50	12	37	2	0
	18	14	500	192	11	1	0
Two colony types in replates	19	28	75	0	3	0	1
	20	28	62	8	38	0	0
	21	28	50	7	2	0	0
	22	30	50	10	1	0	0
	23	21	100	66	0	0	0
	24	14	40	20	13	0	0
	25	14	50	14	1	0	0
	26	28	37	23	0	0	0
One colony type in replates	27	21	150	57	0	0	0
	28	28	50	10	0	0	0
	29	28	15	18	0	0	0
	30	14	100	48	0	0	0
	31	14	50	24	0	0	0
	32	14	50	0	39	0	0
	33	21	63	0	20	0	0
	34	30	30	0	11	0	0
	35	30	48	0	28	0	0
	36	28	24	0	29	0	0
	37	28	100	0	24	0	0
	38	28	25	0	5	0	0
	39	28	25	0	7	0	0
	40	28	37	0	32	0	0

*Blast cell colonies represent samples from 18 different bone marrow donors. This list is intended to demonstrate the range of proliferative potentials of the blast cells in replating experiments and does not show all replates from the 18 donors.

†Culture day is the number of days of primary culture at which time the blast cell colony is plucked for replating.

ary blast cell colonies.⁸ A particular set of culture conditions may select for differentiation over self-renewal. Even though the cells appear morphologically uniform, this is clearly not a reflection of their ability to reclone in secondary cultures (Table 2). Morphological appearance may not reflect the initial stages of differentiation and commitment, and other methods may be necessary to investigate the early stages of differentiation. Unlike leukemic blasts, the blast cells derived from normal tissues are not arrested in maturation, and differentiation should be expected of these cells if they are not merely an artifact of in vitro culture. In this regard, the late blasts observed in our culture system (type I blast of Keller and colleagues⁸) do demonstrate morphological evi-

dence of differentiation and a commitment to granulocytic maturation. The growth of more colonies than blast cells seeded in the replating experiments (Table 2) can be interpreted as a technical error in enumeration of the number of cells contained within the primary colony. Because we observed this on several occasions, we favor the hypothesis that the increase reflects the expansion of the blast cell pool and is consistent with the appearance of secondary blast cell colonies in the replates.

The blast colony progenitor cell appears to be more primitive than the CFU-GEMM in both human and murine hematopoiesis. The blast progenitor cell colony contains only immature cells, gives rise to both additional blast cell colo-

nies as well as mixed granulo-erythroid-megakaryocyte (CFU-GEMM) colonies when replated, and appears at various times after the establishment of culture, suggesting that the cell is in a resting state of cell cycle with a random entry into clonal growth. Only limited numbers of secondary colonies were found in replates of the CFU-GEMM colonies, and no secondary colonies were found in our replates of mature granulocytic or erythroid colonies. Blast cell colonies have not yet been demonstrated to arise from mixed colonies.

The blast cell appears to be functionally different from the more mature progenitor cells. We observed blast cell colonies grown from human bone marrows that have been exposed to 4-hydroperoxycyclophosphamide (4-HC)¹⁸ to "purge" tumor cells prior to autologous bone marrow transplantation.¹⁹ We and others also observed this relative resistance of the blast cell to 4-HC as compared with later progenitors in normal human bone marrow (data not shown) and in murine

spleen²⁰ samples, demonstrating a possible clinical benefit. Because these "purged" bone marrows are capable of reconstituting the hematopoietic system of the transplanted recipients, this blast cell may be the cell responsible for the reconstitution of hematopoiesis following bone marrow transplantation. The functional role of the blast progenitor cell in comparison to the mature progenitor cells is under active investigation at this center.²¹

This cell may represent the human hematopoietic stem cell, and this assay may serve as a fairly simple in vitro means to probe stem cell function. Certainly, this cell will merit further investigation including but not limited to its response to growth regulation and its role in the disordered states of hematopoiesis in humans.

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Normal Synthesis and Expression of Endothelial IIb/IIIa in Glanzmann's Thrombasthenia

By J.C. Giltay, O.C. Leeksa, C. Breederveld, and J.A. van Mourik

Glanzmann's thrombasthenia is a bleeding disorder, inherited in an autosomal recessive way and characterized by an absence or deficiency of the platelet glycoprotein (GP) IIb/IIIa complex. Recently, we and others demonstrated that cultured human umbilical vein endothelial cells synthesize a membrane protein complex similar to the platelet GP IIb/IIIa complex. In this article, we demonstrate that endo-

thelial cells isolated from the umbilical vein of a newborn with Glanzmann's thrombasthenia, as compared with normal endothelial cells, show no difference in their ability to synthesize and express this GP IIb/IIIa complex. Our results indicate that Glanzmann's thrombasthenia is not accompanied by an "endotheliopathy."

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DURING THE LAST few years endothelial cell properties, such as binding of fibrinogen,^{1,2} thrombin,³⁻⁶ and factors V,^{7,8} IX,⁹⁻¹¹ and X¹¹ have been described, suggesting an important role of the endothelial cell plasma membrane, analogous to the platelet membrane, in hemostasis and thrombosis.

In view of this endothelial cell receptor function, parallels were proposed between the endothelial and platelet plasma membrane. We and others demonstrated the synthesis by endothelial cells of a membrane glycoprotein (GP) indistinguishable from platelet GP IIa^{12,13} and of a protein complex similar to the platelet GP IIb/IIIa complex.¹³⁻¹⁵ Recently, endothelial cells were proposed to synthesize a protein immunologically related to platelet GP Ib as well.¹⁶

In Glanzmann's thrombasthenia, an autosomal recessive inherited hemorrhagic disease,^{17,18} the GP IIb/IIIa complex is absent or deficient on the platelet membrane. We wondered, therefore, whether thrombasthenic patients would have a deficiency, not only of the platelet GP IIb/IIIa complex, but also of the endothelial GP IIb/IIIa complex.

To attempt to answer this intriguing question we cultured endothelial cells isolated from the umbilical vein of a newborn with Glanzmann's thrombasthenia. We demonstrated, by immunofluorescence, crossed immunoelectrophoresis (CIE), and immunoprecipitation, that the patient's endothelial cells were indistinguishable from normal endothelial cells in their ability to synthesize and express a GP IIb/IIIa complex.

MATERIALS AND METHODS

Patient. The patient (Y.O., a boy) was born with petechiae and hematomas, despite a normal platelet count ($245 \times 10^9/L$). His parents, who were first cousins, were of Moroccan origin and had two older thrombasthenic children. The diagnosis of Glanzmann's thrombasthenia was based on the following criteria. The patient's platelets failed to aggregate in response to ADP or collagen but did aggregate in response to ristocetin. No binding of CLB-C17 (Table 1) or other anti-GP IIb/IIIa monoclonal antibodies was observed by indirect immunofluorescence or in a whole-blood binding assay.²⁰ No GP IIb/IIIa could be demonstrated by CIE (not shown) and two-dimensional nonreduced/reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig 1).^{12,21}

Endothelial cell culture. Endothelial cells of the patient and those of normal controls were isolated from umbilical cords and cultured as described previously.¹²

Immunofluorescence. Endothelial cells were detached from culture flasks by brief exposure to 0.05% trypsin and 0.02% EDTA (wt/vol) (GIBCO, Paisley, Scotland), followed by neutralization with 300 $\mu g/mL$ of soybean-trypsin inhibitor (Sigma Chemical, St Louis). Cells were centrifuged, washed three times, and fixed with

1% paraformaldehyde as previously described for platelets.²² Binding of monoclonal antibodies and fluoresceine isothiocyanate (FITC)-labeled goat anti-mouse antibodies to either endothelial cells or platelets in suspension was quantitated with a fluorescence-activated cell sorter (EPICS Coulter, Hialeah, FL).

Crossed immunoelectrophoresis. Samples equivalent to 6×10^7 platelets or 1×10^6 endothelial cells were run against 3% and 1.2% (vol/vol) polyclonal rabbit antiplatelet antiserum, respectively, and an intermediate gel containing ¹²⁵I-labeled CLB-C17 IgG.^{12,14,20} Slides were stained with Coomassie brilliant blue and subsequently subjected to autoradiography.

Immunoprecipitation. Immunoprecipitation was performed as reported,¹² using a preformed complex of monoclonal antibodies and goat anti-mouse antibodies. Labeling procedures with ³⁵S-methionine and ¹²⁵I were as described,¹² with the exception that metabolic labeling with ³⁵S-methionine was not performed with endothelial cells on microcarriers but in 75-cm² culture flasks.

RESULTS

Immunofluorescence. CLB-C17, a monoclonal antibody directed against an epitope on the platelet and endothelial GP IIb/IIIa complex,^{12,19} bound to the same extent to the patient's endothelial cells as to control cells (Table 1), whereas no binding to the patient's platelets was observed. Values obtained with Glanzmann's thrombasthenia and concurrent control endothelial cells using CLB-C17 and CLB-HEC-75 (an anti-GP IIa antibody¹² that served as a positive control) were well within the normal range (63.4 ± 12.0 , 86.0 ± 4.3 , respectively, mean $\pm$ SD, $n = 5$).

Crossed immunoelectrophoresis. By CIE of an endothelial cell lysate against a polyclonal antiplatelet antiserum and ¹²⁵I-labeled CLB-C17, one precipitation arc could be visual-

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Table 1. Binding of Monoclonal Antibodies to Endothelial Cells and Platelets in Suspension as Determined by Indirect Immunofluorescence

Subject	Platelets			Endothelial Cells		
	CLB-C17	CLB-HEC-75	NMS	CLB-C17	CLB-HEC-75	NMS
Glanzmann's thrombasthenia (patient Y.O.)	0.9	96.3	3.1	45.1	81.8	7.6
Control	92.1	93.6	1.7	57.3	89.2	18.0

NMS, normal mouse serum.

The numbers are percentages of platelets or endothelial cells showing a positive reaction (ie, above background). CLB-C17 recognizes an epitope on the glycoprotein (GP) IIb/IIIa complex.^{14,19} CLB-HEC-75 is an anti-GP IIa antibody¹² used as a positive control.

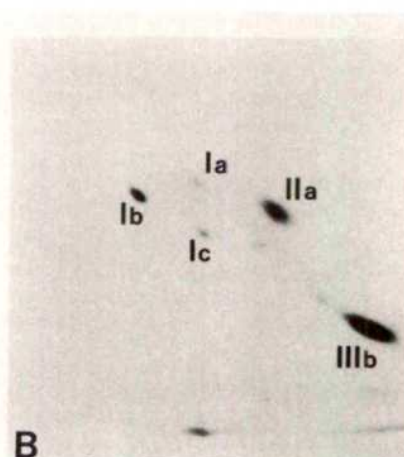
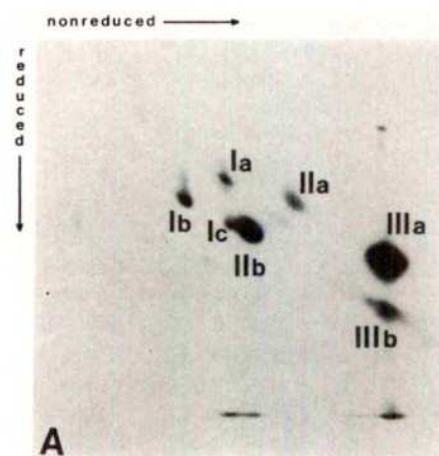


Fig 1. Analysis of platelet membrane glycoproteins (GPs) by nonreduced/reduced two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Autoradiogram of normal platelets (A) and the platelets of patient Y.O. (B). GP IIb and GP IIIa are absent in (B), whereas the other major GPs are present.

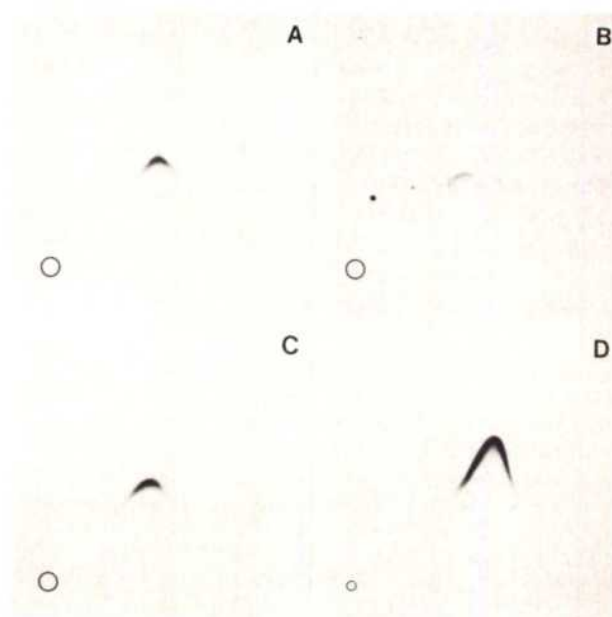


Fig 2. Crossed immunoelectrophoresis of Triton X-100 lysates of endothelial cells and platelets against a polyclonal antiplatelet antiserum. (A) Autoradiogram of normal endothelial cells; (B) autoradiogram of thrombasthenic endothelial cells; (C) autoradiogram of a mixture of normal and thrombasthenic endothelial cells, and (D) autoradiogram of normal platelets.

ized after autoradiography, using either a lysate of thrombasthenic cells (Fig 2B) or control cells (Fig 2A). To prove that both precipitation arcs had the same mobility, CIE of a mixture of both cell lysates was performed and again revealed one precipitation arc (Fig 2C).

Immunoprecipitation. CLB-C17 precipitated two proteins from lysates of metabolically (³⁵S methionine) as well as surface (¹²⁵I)-labeled thrombasthenic endothelial cells (Fig 3A, lanes 3 and 7). These proteins had the same mobility and were of an intensity equal to those precipitated from lysates of control cells (Fig 3A, lanes 2 and 6).

Antibodies against the platelet alloantigen Zw^a (PI^{A1}; located on GP IIIa,^{23,24} deficient on thrombasthenic platelets,^{25,26} and recently shown to be present on endothelial cells^{27,28}) gave precipitation results similar to those of CLB-C17 (not shown), indicating normal expression of the Zw^a (PI^{A1}) antigen by these thrombasthenic endothelial cells.

DISCUSSION

The endothelial cells isolated from the umbilical vein of our patient with Glanzmann's thrombasthenia, synthesize and express a protein complex that is qualitatively and quantitatively indistinguishable from the GP IIb/IIIa complex synthesized and expressed by normal endothelial cells. These findings indicate that Glanzmann's thrombasthenia is not accompanied by an "endotheliopathy," and are in keeping with the view of Nurden and colleagues²⁹ that the

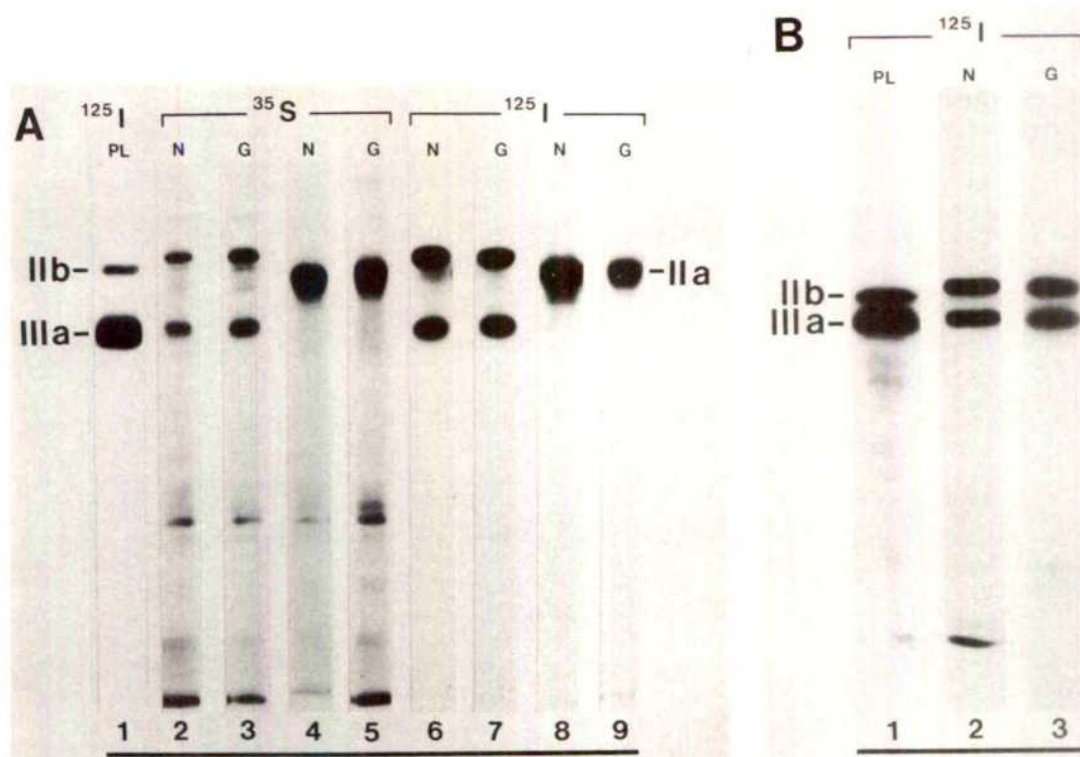


Fig 3. Immunoprecipitation of endothelial cells either metabolically labeled with ^{35}S -methionine or surface-labeled with ^{125}I . (A) Nonreduced: lane 1, as a reference, ^{125}I -labeled IIb and IIIa precipitated with CLB-C17 from normal platelets (PL); lane 2, ^{35}S -labeled proteins precipitated with CLB-C17 from normal endothelial cells (N); lane 3, as in lane 2, using thrombasthenic endothelial cells (G); lane 4, ^{35}S -labeled IIa isolated with CLB-HEC-75 from normal endothelial cells; lane 5, as in lane 4, using thrombasthenic endothelial cells; lane 6, ^{125}I -labeled proteins precipitated with CLB-C17 from normal endothelial cells; lane 7, as in lane 6, using thrombasthenic endothelial cells; lane 8, ^{125}I -labeled IIa isolated with monoclonal antibody CLB-HEC-75 from normal endothelial cells; lane 9, as in lane 8, using thrombasthenic endothelial cells. (B) Reduced: lane 1 corresponds with lane 1 in A; lane 2 corresponds with lane 6 in A; and lane 3 corresponds with lane 7 in A.

structural genes coding for GP IIb and IIIa in this disease have no major defect. The platelet defect may be caused by a defect in a regulatory element affecting the transcription of both genes in megakaryocytes, as suggested by Bray and co-workers.³⁰

Platelet and endothelial GP IIb/IIIa are not identical, however. As shown previously^{13,14} and in this study, the electrophoretic mobility of the complexes and their subunits is different, as judged by CIE (Fig 2) and SDS-PAGE (Fig 3). Furthermore, not all of the monoclonal antibodies directed against platelet GP IIb/IIIa crossreact with endothelial GP IIb/IIIa,^{14,31} and, finally, in contrast to platelet GP IIb/IIIa, the endothelial complex cannot be dissociated by treatment with EDTA.^{13,14} Thus, although the endothelial GP IIb/IIIa complex is recognized by a number of anti-

platelet IIb/IIIa antibodies (including antibodies directed against the platelet alloantigen Zw^a (Pl^{A1})),^{27,28} structural differences are apparent.

Taking these findings together, we propose that the endothelial GP IIb/IIIa-like complex is encoded by genes that are similar but not identical to the genes coding for platelet GP IIb/IIIa. In this context, platelet GP IIb/IIIa seems to be structurally related to cell surface GPs of other cells, including lymphocytes and monocytes, and with these constitutes a family of molecules.³²⁻³⁴ The endothelial GP IIb/IIIa complex may belong to this family. More definite answers to these questions will be obtained by cloning the GP IIb/IIIa gene, allowing study at the DNA level to establish the structural features of the genes coding for the endothelial and platelet GP IIb/IIIa.

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Human Neutrophil Elastase Alters Human α -Thrombin Function: Limited Proteolysis Near the γ -Cleavage Site Results in Decreased Fibrinogen Clotting and Platelet-Stimulatory Activity

By Mark S. Brower, Daniel A. Walz, Kimberly E. Garry, and John W. Fenton II

During blood coagulation, polymorphonuclear leukocytes release elastase in amounts that can exceed 100 nmol/L. We therefore studied the interaction between human leukocyte elastase and human α -thrombin. Elastase cleaved the thrombin B chain (Ala 150-Asn 151) near the γ -cleavage site, resulting in two fragments held together by noncovalent interactions. The NH_2 -terminal fragment (FI), mol wt ~18,000, was disulfide-linked to the thrombin A chain. The COOH-terminal fragment (FII), mol wt ~13,000, contained the active-site serine and formed a covalent bond with antithrombin III. Heparin accelerated proteolysis of α -thrombin by elastase. Proteolyzed α -

thrombin (T_α) retained full amidolytic activity; however, the concentration of T_α causing 50% maximal platelet aggregation and adenosine triphosphate (ATP) release was 7.9 nmol/L (1.1 nmol/L for α -thrombin and 220 nmol/L for γ -thrombin). Fibrinogen clotting activity of T_α and γ -thrombin was 32% and 1% that of α -thrombin, respectively. Elastase released during the coagulation process may modulate thrombin activity. In addition, elastase-modified thrombin may be a useful probe of the structure and function of the γ -cleavage region.

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ELASTASE IS the major neutral protease released by human polymorphonuclear leukocytes (PMNs)¹ during blood coagulation or activation by soluble immune complexes, C5a, or endotoxin.¹⁻³ Elastase is released into the circulation in acute leukemia, in septicemia,⁴ and in a variety of other inflammatory conditions.³ Several studies suggest that the decrease in the concentration of coagulation factors that occurs in these conditions may be the result of direct proteolysis by PMN elastase.^{5,6} Elastase also inactivates human antithrombin III,⁷ α_2 -plasmin inhibitor, and C1 inactivator.⁸ Furthermore, it inhibits thrombin-induced platelet stimulation in association with limited proteolysis of platelet membrane glycoprotein Ib.⁹ In circulating blood, leukocyte elastase is inactivated mainly by α_1 -antitrypsin¹⁰; however, this inhibitor is readily inactivated by oxidizing agents such as those generated by phagocytosing PMNs.^{11,12} More recently, studies using cell contact systems have shown that α_1 -antitrypsin does not completely inhibit the proteolytic activity of PMN-derived elastase.^{13,14}

In this study, we investigated the effects of PMN elastase on human α -thrombin. PMN elastase cleaved the thrombin B chain at Ala 150-Asn 151, within four amino acids of the γ -cleavage site. Cleavage resulted in formation of a larger NH_2 -terminal and a smaller COOH-terminal fragment held together by noncovalent association. The elastase-modified thrombin possessed amidolytic activity similar to that of α -thrombin but exhibited reduced fibrinogen clotting and platelet-stimulatory activity. Elastase degradation of α -thrombin was enhanced by heparin. These findings suggest that elastase may be involved in the physiologic modulation of α -thrombin function.

MATERIALS AND METHODS

Human polymorphonuclear leukocyte elastase, specific activity 92%, was purified from outdated WBC concentrates or from purulent sputum.^{8,15} Human α -thrombin and γ -thrombin were prepared and stored at -70°C in 0.75 mol/L of NaCl at concentrations of 2 to 4 mg/mL.^{16,17} As determined by active-site titration (*p*-nitrophenyl-*p'*-guanidinobenzoate), the specific activities of the α -thrombin and γ -thrombin preparations were 95% and 78%, respectively.^{18,19} Antithrombin III was obtained as a gift from the American Red Cross Fractionation Center, Bethesda, MD. As analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),

the inhibitor migrated as a single band; >96% of the antithrombin III formed a higher mol-wt complex with α -thrombin.

Proteolysis of α -thrombin by elastase. α -Thrombin (~10 $\mu\text{mol/L}$) was incubated with elastase (thrombin/elastase molar ratios from 1:1 to 100:1) for various times (37°C) in 0.02 mol/L of Trizma base (Tris), pH 7.4, or in Tris buffer containing 0.15 mol/L of NaCl. Incubations were performed in the presence or absence of 1 U/mL of sodium heparin (140 U/mg) from porcine intestine (Elkins-Sinn, Cherry Hill, NJ). The reaction was stopped either by incubating for 10 minutes (37°C) with 0.7 mmol/L of MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl (Enzyme Systems Products, Livermore, CA) to inhibit elastase activity²⁰ or by boiling in SDS as detailed later. Samples were quantitated for residual elastase activity with MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl (Enzyme Systems Products) as described.²¹ Only those samples demonstrating >95% inhibition of initial activity were used in the following studies.

SDS-PAGE. Samples for electrophoresis (4 to 8 μg per lane) were mixed with an equal volume of 0.06 mol/L of Tris, 2% SDS, 5% glycerol, 0.001% bromphenol blue, and 2% dithiothreitol (SDS-DTT), solubilized by heating at 100°C for 3 minutes, and stored (-70°C). SDS-PAGE was performed by the method of Laemmli,²² using a 3.8% stacking gel and a 9% to 18% gradient separation gel. Electrophoresis was terminated when the buffer front was 1 cm from the bottom of the gel. Gels were fixed in 25% isopropanol and 10%

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acetic acid prior to being stained with Coomassie blue (0.1%). Densitometry was performed at 633 nm using an LKB Ultrascan Laser Densitometer and LKB model 2210 dual-channel recorder.

Mol wt markers included phosphorylase b (mol wt 94,000), bovine serum albumin (BSA) (mol wt 67,000), ovalbumin (mol wt 43,000), carbonic anhydrase (mol wt 30,000), soybean trypsin inhibitor (mol wt 20,100), α -lactalbumin (mol wt 14,400), and aprotinin (mol wt 6,500).

Interaction of elastase-modified thrombin with antithrombin III. α -Thrombin (11 μ mol/L) was incubated either alone or with elastase (1.7 μ mol/L) for 60 minutes (37 °C) in 0.02 mol/L of Tris pH 7.4 containing 1 U/mL of porcine intestinal heparin. The mixture was incubated for 10 minutes (37 °C) with 0.7 mmol/L of MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl prior to addition of antithrombin III (30 μ mol/L). Samples were taken at various times for analysis by SDS-PAGE.

Amino acid sequence of elastase-modified thrombin. Samples were prepared for sequencing as described²³ by extensive dialysis against 0.1% trifluoroacetic acid (TFA) and freeze-drying. The final thrombin product (210 μ g) was dissolved in 0.1% TFA, and one-half (2.6 nmol) was applied to a Beckman 890M spinning cup sequencer. The resultant phenylthiohydantoin (PTH) derivatives were resolved on a Beckman Ultrasphere 5- μ m ODS column using a Waters 501 pump, 440 detector (254 nm) and 740 integrating recorder. Isocratic separation of the common PTH amino acids was achieved at 55 °C with 0.01 mol/L of sodium acetate, pH 4.54 and acetonitrile (38:62, vol/vol) at a flow rate of 1 mL/min. PTH yields were automatically calculated by peak area and expressed as a percentage of the respective 200-pmol standard. Duplicate thrombin samples were subjected to sequence analysis.

Amidolytic activity. Samples of α -thrombin and elastase-treated thrombin (>95% conversion to the cleaved form on SDS-PAGE) were analyzed for amidolytic activity with Tos-Gly-Pro-Arg-paranitroanilide (GPA, Vega) by modification of the method of Kirchoff and colleagues.²⁴ GPA was dissolved in methanol (14 mg/mL). Duplicate dilutions of thrombin (0.3 to 270 nmol/L) in Tris 0.2 mol/L, pH 7.4 containing 1 mg/mL of BSA (Sigma) were incubated at 23 °C (final volume 0.2 mL per well) with GPA (0.5 mg/mL) in 96-well microtitration plates (Nunc). Color development at 405 nm was followed on a Titertek Multiscan photometer (Flow Laboratories, McLean, VA). The change in absorbance per minute was subtracted from the values obtained in wells that did not contain thrombin. Plots of color development with time demonstrated a linear relationship.

Fibrinogen clotting activity. Human fibrinogen (Kabi Grade L) was dissolved in 0.3 mol/L of NaCl, 0.1 mol/L of sodium citrate pH 6.4, containing 0.01 mol/L of ϵ -aminocaproic acid (Aldrich, Milwaukee), and stored (–70 °C). For thrombin assays, fibrinogen was diluted (6.4 mg/mL) in 10 mmol/L of imidazole, 10 mmol/L of CaCl₂, 0.15 mol/L of NaCl pH 7.4. Aliquots (0.09 mL) were prewarmed to 37 °C prior to the addition of various concentrations of thrombin prepared in 0.2 mL of the same buffer (37 °C) containing 0.1% BSA. Clotting time was determined by fibrometer (Becton Dickinson No. 60415).

Platelet stimulation studies. Washed platelet suspensions were prepared by modification of the technique of Mustard and co-workers as described.^{6,25-27} Platelet stimulation studies were performed at 37 °C with 0.45-mL aliquots of washed platelets (2×10^8 /mL) stirred at 1,000 rpm in a Chrono-Log model 460 Lumi-Dual double-channel aggregometer. Aggregation and luminescence in each channel was recorded with a Chrono-Log model 703 strip chart dual-pen recorder. Aggregation was quantitated by planimetry.²⁸ Results were expressed as percentage of the area under the control curves.

Secretion was measured by the luminescence reaction of secreted

ATP with 4 μ L of firefly luciferin and luciferase (40 mg/mL, Chrono-Lume No. 395, Chrono-Log) added 1 to 5 minutes prior to stimulation of the platelets.^{29,30} Secretion was measured as the maximum increase in fluorescence and expressed as the percentage of the increase in the control.

The threshold stimulus determined for individual platelet preparations was defined as the lowest concentration that caused $\geq 90\%$ increase in light transmission at 5 minutes when added to stirred platelets at 37 °C³⁰ and resulted in fluorescence at least 90% of maximum. Immediately after measurements of aggregation and secretion, 40 μ mol/L of ATP (Sigma FF-ATP, 1 mg/mL, and 40 mg/mL of MgSO₄ · 7 H₂O) was added for instrument calibration.

RESULTS

Incubation of α -thrombin with elastase. α -Thrombin was incubated alone or with elastase for various times (37 °C). After MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl was added to inhibit elastase activity, samples were reduced, electrophoresed, and stained with Coomassie blue. The B chain of thrombin migrated as a single band at mol wt 32,500 after incubation for 0 to 30 minutes with buffer alone (Fig 1, lanes 9 through 10). The small (mol wt) α -thrombin A chain was not visualized in this system.¹⁶ Incubation of thrombin with elastase (thrombin/elastase molar ratio 8:1) resulted in proteolysis (lanes 1 through 4). After incubation with elastase for 15 seconds, lower mol wt bands were observed (lane 1). Those at mol wt ~28,000 co-migrated with the elastase isomers³¹ seen in the elastase controls (lanes 11 and 12). New bands also appeared at mol wt 21,000, 16,000, and 15,000. These bands were diminished or absent if elastase was inactivated with α_1 -antitrypsin (experiment not shown) or with greater concentrations of MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl prior to the addition of SDS to the mixture. This suggests that these peptides may result from either (a)

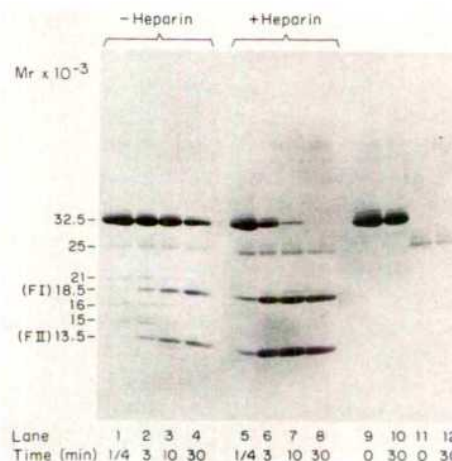


Fig 1. Effect of heparin on thrombin proteolysis. α -Thrombin (10 μ mol/L) was incubated (37 °C) with elastase (1 μ mol/L) in 0.02 mol/L of Tris pH 7.4 with (lanes 1 through 4) or without (lanes 5 through 8) of heparin (1 U/mL). After various times, MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl (0.7 mmol/L) was added to inhibit elastase activity. Samples were electrophoresed (5 μ g thrombin per lane) and stained with Coomassie blue. The α -thrombin controls at 0 and 30 minutes are shown in lanes 9 and 10, respectively; those for elastase are shown in lanes 11 and 12.

elastase-proteolysis of SDS-denatured thrombin or (b) stimulation of elastase activity by SDS.³²⁻³⁴

With longer incubations (lanes 2 through 4), the concentration of thrombin B chain at mol wt 32,500 was diminished; by 30 minutes proteolysis of 50% of the thrombin B chain occurred. The three bands at mol wt 21,000, 16,000, and 15,000 were no longer apparent after 30 minutes, and two additional bands representing core fragments appeared at mol wt 18,500 and 13,500, designated fragment I (FI) and fragment II (FII), respectively. In four separate experiments, the mol wt of FI ranged from 17,000 to 18,500, and that of FII ranged from 12,000 to 13,500.

In experiments not shown, FI and FII could not be separated under nondenaturing conditions (0% to 1 mol/L of NaCl; 0% to 3% Triton X-100, Sigma, St Louis). This suggests that FI and FII are held together by strong noncovalent interactions, as reported for the γ -thrombin B chain fragmentation peptides.³⁵⁻³⁸

Heparin binds to both thrombin and elastase.³⁹⁻⁴³ Therefore, the effect of heparin on elastase-mediated proteolysis of α -thrombin was examined. α -Thrombin was incubated with elastase in Tris buffer with 1 U/mL of heparin (lanes 5 through 8). Within 15 seconds after the addition of elastase to α -thrombin, FI and FII were observed (lane 5). The breakdown products at mol wt 21,000, 16,000, and 14,000 seen in lanes 1 and 2 were not observed in the presence of heparin. Within 3 minutes (lane 6), >50% of α -thrombin was proteolyzed to FI and FII; by 30 minutes (lane 8), the conversion of α -thrombin to FI and FII was complete.

These results indicate that heparin accelerates elastase-mediated proteolysis of α -thrombin. They also suggest that heparin protects α -thrombin from conversion into the mol wt 21,000, 16,000, and 14,000 fragments.

Elastase cleavage of factor IX is less extensive in the absence of calcium.⁶ Therefore, the above experiments were performed in the presence of CaCl_2 (2 to 6 mmol/L). Calcium did not alter or inhibit proteolysis of thrombin by elastase.

When analyzed on SDS-PAGE without disulfide bond reduction, thrombin fragments FI and FII were identified at mol wt 23,000 and 13,500 (gel not shown). The increase in the mol wt of FI prior to reduction suggested that it was covalently linked to the thrombin A chain.

Interaction of elastase-modified thrombin with antithrombin III: Identification of the active-site-containing fragment. To determine if FI or FII contained the active-site serine, antithrombin III was incubated either with α -thrombin or with the elastase-modified protease in the presence of 1 U/mL of heparin (inhibitor/enzyme molar ratio ~3:1). After various times, samples were analyzed by SDS-PAGE (Fig 2). α -Thrombin incubated in the presence of elastase (lane 1) was converted into the elastase-modified form, as indicated by the presence of FI and FII. A faint band representing elastase was seen at mol wt 28,000. Lane 2 contained α -thrombin incubated in the absence of elastase for comparison, and lane 3 contained antithrombin III (mol wt 58,000). Incubation of antithrombin III with the modified thrombin for 15 seconds, 5 minutes, and 60 minutes (lane 4, 5, and 6, respectively) resulted in formation of a band at mol

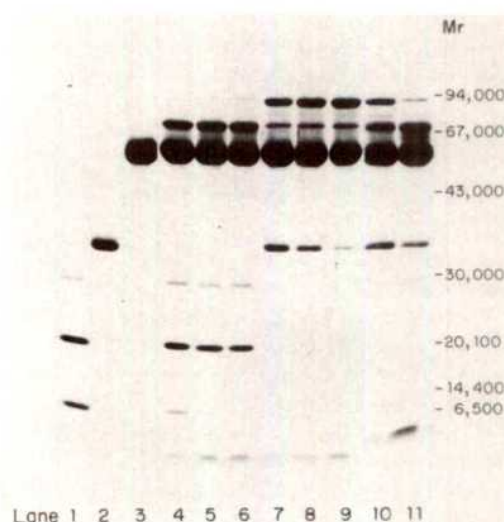


Fig 2. Interaction of elastase-modified thrombin with antithrombin III. α -Thrombin (11 $\mu\text{mol/L}$) was incubated either alone or with 1.7 $\mu\text{mol/L}$ of elastase at 37 $^{\circ}\text{C}$ prior to incubation at 37 $^{\circ}\text{C}$ (10 minutes) with MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl (0.7 mmol/L). Antithrombin III (30 $\mu\text{mol/L}$) was added to the mixtures, and samples were taken for analysis at various times. Lane 1, elastase-modified α -thrombin; lane 2, α -thrombin control; lane 3, antithrombin III control; lanes 4 through 6, incubation of antithrombin III with elastase-modified thrombin shown in lane 1 for 15 seconds, 5 minutes, and 60 minutes, respectively; lanes 7 through 9, incubation of antithrombin III with α -thrombin shown in lane 2 for 15 seconds, 5 minutes, and 60 minutes, respectively; lanes 10 and 11, α -thrombin was incubated with antithrombin for 5 minutes as in lane 8 prior to the addition of elastase (1.7 $\mu\text{mol/L}$). Samples were taken at 15 seconds (lane 10) and 5 minutes (lane 11). Positions of the mol wt markers are indicated on the right.

wt 72,000, the mol wt compatible with that of a complex between antithrombin III (mol wt 58,000) and FII (mol wt 13,500). In addition, after 15-second incubation (lane 4) the intensity of free FII was diminished relative to FI; within 5 minutes of incubation (lane 5), FII was no longer apparent. These findings suggest that the active-site serine is contained within FII.

For comparison, antithrombin III was incubated with α -thrombin for 15 seconds, 5 minutes, and 60 minutes, respectively (lanes 7 through 9). The α -thrombin-antithrombin III complex was visible at mol wt 88,500. A faint band was apparent at mol wt ~72,000, with electrophoretic mobility similar to the FII-antithrombin III complex. This mol wt ~72,000 complex has been shown to be the result of degradation of the α -thrombin-antithrombin complex by free thrombin.^{44,45} The relationship of this complex to the FII-antithrombin complex remains undetermined.

These results demonstrate that antithrombin III inhibits elastase-modified thrombin by combining with the active-site serine within FII. These findings suggest that elastase cleaves the α -thrombin B chain in the vicinity of the γ -cleavage site.⁴⁶ This results in the formation of an NH₂-terminal fragment (FI), mol wt 18,000, that is disulfide linked to the thrombin A chain and a COOH-terminal fragment (FII), mol wt 13,000, containing the active-site serine.

To determine if elastase can degrade preformed α -thrombin-antithrombin complexes, thrombin was incubated with antithrombin for 5 minutes as in lane 8 prior to incubation with elastase (thrombin/elastase molar ratio 9:1). Fifteen seconds after addition of elastase (lane 10), a complex was observed at mol wt 72,000; by 5 minutes (lane 11), the α -thrombin-antithrombin complex at mol wt 88,500 was markedly reduced in intensity, and protein at mol wt 72,000 was increased. A faint band migrated at mol wt 16,000. Neither fragment F1 nor fragment FII was observed in lanes 10 or 11, and there was an increase in stained material moving just behind the buffer front. These findings suggest that elastase cleavage of the α -thrombin-antithrombin complex may differ from cleavage of free α -thrombin.

Amino acid sequence of elastase-modified thrombin. Because the thrombin samples were analyzed directly after proteolysis, at least one new amino terminal sequence was anticipated, in addition to the usual A chain and B chain sequences. Amino acid analysis confirms the presence of a new NH_2 -terminal sequence corresponding to residues 151 through 156 of the human B chain of thrombin (Table 1). Residues 1 (Asn), 4 (Lys), and 6 (Gln) are unequivocal; the yields at residues 2 (Val), 3 (Gly), and 5 (Gly) are in excess of theoretical yields, assuming that only one or two peptides exist. Further efforts are under way to resolve these components so that peptide yields and the extent, if any, of protein fragmentation at the carboxy terminal regions of the three constituent polypeptides detected there can be quantitatively determined.

Amidolytic activity of elastase-modified thrombin. Increasing concentrations of elastase-modified thrombin were added to microtitration plate wells containing GPA (Fig 3). Color generation was proportional to the concentration of enzyme in the range of 13.6 to 272 nmol/L. The amidolytic activity of the modified enzyme appeared to be nearly identical to that of α -thrombin.

Fibrinogen clotting activity of elastase-modified thrombin. The fibrinogen clotting activity of elastase-modified thrombin (>98% conversion to the modified form in the presence of heparin 1 U/mL) was compared with that of α - and γ -thrombin (Fig 4). At each thrombin concentration, the clotting time of the modified thrombin was greater than that

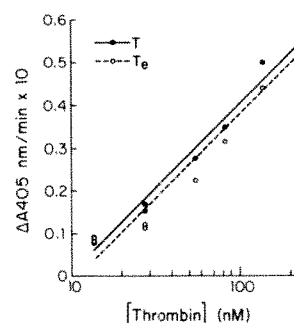


Fig 3. Amidolytic activity of elastase-modified thrombin. α -Thrombin [T] or elastase-modified thrombin (T_e) was diluted (0.3 to 270 nmol/L) in buffer and incubated at 23 °C with Tos-Gly-Pro-Arg-paranitroanilide (GPA) (0.5 mg/mL). Color development was analyzed as detailed in the Materials and Methods section. Each point is the average of duplicate determinations. Curves were determined by linear regression analysis. For T, $y = 39.2x - 33.2$ ($r = 0.988$); for T_e , $y = 39.7x - 41.4$ ($r = 0.968$).

of α -thrombin but less than that of γ -thrombin. As determined by comparing the slopes of the three lines obtained by linear regression analysis (Fig 4 legend), elastase-modified thrombin was 32% as potent as α -thrombin in clotting fibrinogen; γ -thrombin was 1% as potent as α -thrombin. The relative slopes of the three curves were unaltered by the presence or absence of heparin or by the addition of elastase to α -thrombin immediately prior to mixing with fibrinogen (experiments not shown).

Platelet stimulatory activity of elastase-modified thrombin. To compare the platelet-stimulatory activity of elastase-modified thrombin with that of α -thrombin and γ -

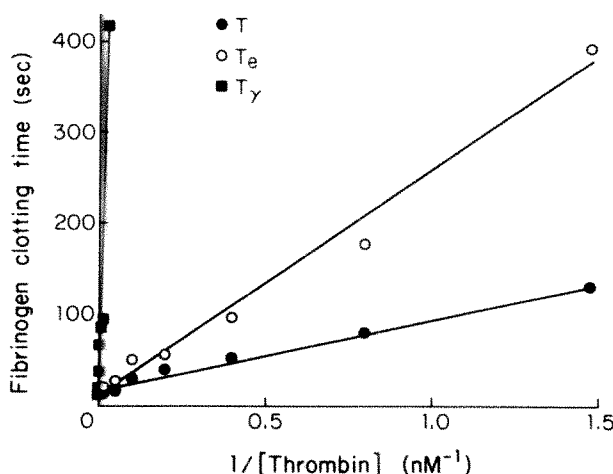


Fig 4. Fibrinogen clotting activity of elastase-modified thrombin. α -Thrombin (T), elastase-modified thrombin (T_e), or γ -thrombin (T_γ) was diluted in buffer and prewarmed to 37 °C. After fibrinogen was added (2.0 mg/mL), fibrin clotting time was determined as detailed in the Materials and Methods section. Each point represents an individual clotting time measurement. Curves were determined by linear regression analysis. For T, $y = 77.7x + 17.02$ ($r = 0.968$); for T_e , $y = 245.6x + 8.66$ ($r = 0.992$); and for T_γ , $y = 6629x + 12.44$ ($r = 0.941$). Study shown is representative of studies on four separate T_e preparations. Equivalent concentrations of T, T_e , and T_γ had similar amidolytic activities against Tos-Gly-Pro-Arg-paranitroanilide (GPA).

Table 1. Sequence of Elastase-Treated Human α -Thrombin

Cycle	Amino Acid Detected		
	A-chain	B-chain	Additional Peptide
1	Thr (0.6)	Ile (1.2)	Asn (0.8)
2	Phe (1.0)	Val (1.9)	Val (1.9)
3	Gly (1.6)	Glu (0.5)	Gly (1.6)
4	Ser (NQ)	Gly (0.7)	Lys (0.8)
5	Gly (1.4)	Ser (NQ)	Gly (1.4)
6	Glu (0.6)	Asp (0.4)	Gln (0.3)

Human α -thrombin (2.6 nmol) was subjected to sequence analysis. Yields (in parentheses) are corrected to final yield, expressed in nanomoles. The sequence, Additional Peptide, corresponds to residues 151 through 156 of the human thrombin B-chain.

Thr, threonine; Ile, isoleucine; Asn, asparagine; Phe, phenylalanine; Val, valine; Gly, glycine; Glu, glutamic acid; Ser, serine; Lys, lysine; Gln, glutamine, NQ, not quantitated.

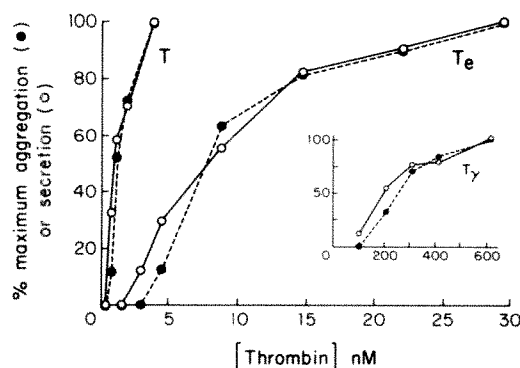


Fig 5. Platelet stimulation by elastase-modified thrombin. Washed platelets (2×10^8 /mL) were stirred in the presence of luciferin and luciferase for 1 minute at 37 °C prior to the addition of elastase-modified thrombin (T_e), α -thrombin (T), or γ -thrombin (T_γ , inset). Aggregation was recorded as the increase in light transmission and quantitated by planimetry. Results were expressed as percentage of the area under the control curves. Secretion was expressed as the maximum increase in fluorescence expressed as a percentage of the increase in the control. Equivalent concentrations of T , T_e and T_γ had similar amidolytic activity against Tos-Gly-Pro-Arg-paranitroanilide (GPA).

thrombin, platelet aggregation and secretion were measured simultaneously with a lumi-aggregometer. Examples of standard curves of α -thrombin-induced platelet aggregation and ATP-mediated luminescence are shown in Fig 5. The concentration of α -thrombin required to liberate 50% of maximally releasable ATP from platelets was 1.1 nmol/L. This was similar to the concentration required to cause 50% of maximal aggregation. In contrast, the concentration of modified thrombin required to liberate 50% of releasable ATP and to cause 50% of maximal aggregation was ~7.9 nmol/L. As a result, both curves representing aggregation and secretion were shifted to the right. Using the concentration required to produce 50% maximal stimulation for comparison, the modified thrombin was ~14% as potent as α -thrombin in stimulating both platelet aggregation and platelet release. In three additional experiments using three different preparations of the modified thrombin, the modified thrombin was 10% to 19% as potent as α -thrombin.

γ -Thrombin was a less potent platelet stimulus than elastase-modified thrombin (Fig 5 inset). The concentration required to cause 50% of maximum platelet aggregation and release was ~220 nmol/L (0.5% as potent as α -thrombin).

Elastase has been reported to inhibit thrombin-induced platelet aggregation and secretion.⁹ The final concentration of elastase in the cuvette (≤ 3.5 nmol/L), however, was less than that required to inhibit platelet function in this system.⁹ In addition, incubation of similar concentrations of chloromethyl ketone inhibited elastase (95% inhibited by MeO-Suc-Ala-Ala-Pro-Val-CH₂Cl) with α -thrombin (0 to 60 minutes) did not inhibit thrombin-induced platelet aggregation or secretion.

DISCUSSION

These studies demonstrate that human α -thrombin, upon proteolysis by PMN elastase, exhibits diminished fibrinogen

clotting activity and diminished platelet stimulatory activity, as measured by platelet aggregation and platelet ATP release. Loss of α -thrombin function is associated with limited cleavage of the thrombin B chain.

The concentration of prothrombin in plasma is sufficient to generate ~150 U/mL of α -thrombin.^{46,47} In addition to being inactivated by circulating inhibitors, α -thrombin selectively binds to fibrin, thereby becoming less susceptible to inactivation by its plasma inhibitors.⁴⁸⁻⁵⁰ These studies suggest that fibrin acts as a reservoir for enzymatically active thrombin. Human PMNs release elastase as blood clots^{2,3}; in addition, PMNs penetrate preformed blood clots, accumulate within thrombin, and ingest fibrin.⁵¹⁻⁵³ Recently, Weitz and co-workers documented that the fibrinogen peptide A α 1-21 is a specific in vivo indicator of elastase-mediated proteolysis of fibrinogen and higher levels of A α 1-21 were found in patients with α_1 -antiprotease deficiency than in healthy controls.⁵⁴ In a similar manner, the limited digestion of α -thrombin by elastase could be an important physiologic mechanism for regulation of the activity of both free and fibrin-bound thrombin.

PMN elastase cleaves the thrombin B chain at Ala 150-Asn 151. This site is four amino acids to the NH₂-terminal side of the autoproteolytic or tryptic γ -thrombin cleavage site; the β domain is not cleaved. Proteolysis by PMN elastase occurs in areas rich in apolar amino acids.^{32,55-57} In contrast to the positively charged thrombin β cleavage domain, the γ cleavage site (Lys 154-Gly 155) resides within a domain containing several apolar amino acids.³⁶⁻³⁸ This area should be susceptible to cleavage by elastase.^{32,55-57} These considerations are compatible with our findings that the larger NH₂-terminal fragment, FI (mol wt ~18,000) is disulfide linked to the thrombin A chain; and the smaller COOH-terminal fragment, FII (mol wt ~13,000) contains the active-site serine, as indicated by the recovery of a covalent bond between FII and antithrombin III. These results are supported by the recent report that porcine pancreatic elastase also cleaves a single peptide linkage in prothrombin corresponding to Ala 150-Asn 151 within the α -thrombin B chain, thereby facilitating localization of the staphylocoagulase-binding region to the COOH-terminal fragment.⁵⁸

PMN elastase binds to glycosaminoglycans of biologic importance, including heparin, heparin sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronic acid.⁴³ Although glycosaminoglycans inhibit the amidolytic activity of elastase against a small oligopeptide substrate,⁴³ heparin stimulates elastase-mediated proteolysis of elastin.⁵⁹ Thus, our finding that heparin accelerates elastase-mediated proteolysis of α -thrombin is not without precedent. It also suggests that the physiologic interaction of elastase with thrombin might be a surface-mediated phenomenon. Finally, because heparin binds to both thrombin and elastase,^{43,60} a template mechanism similar to that for the thrombin-antithrombin III reaction is implied, whereby the interaction of both thrombin and elastase with heparin are involved in the expression of catalytic activity.⁶²⁻⁶³

Elastase-modified thrombin might be an important probe of the thrombin γ cleavage domain. In marked contrast to

autolytic or tryptic digestion of α -thrombin,^{16,44} elastase-mediated proteolysis of the γ region does not require proteolysis near the β cleavage site prior to cleavage in the γ region. Cleavage at the β site reduces fibrinogen clotting activity.⁶⁴⁻⁶⁷ Therefore, it is not surprising that the elastase-modified enzyme, with an intact β domain, retains some fibrinogen clotting activity. Likewise, we found (Fig 5) that the platelet stimulatory activity of elastase-modified thrombin (14% that of α -thrombin) is greater than that of γ -thrombin (0.5% that of α -thrombin). These findings, confirming the results of others for human γ -thrombin,⁶⁸ suggest

that thrombin-induced fibrinogen clotting and platelet stimulation are altered by cleavage near the γ cleavage site. In contrast, cleavage near the γ cleavage site does not appear to inhibit inactivation by antithrombin III.⁶⁹ The limited digest of α -thrombin by elastase should prove useful in the investigation of the structure and function of human thrombin.

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Variability of the Erythropoietic Response in Autoimmune Hemolytic Anemia: Analysis of 109 Cases

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One hundred-nine cases of autoimmune hemolysis were reviewed to determine the frequency of reticulocytopenia, the state of the erythroid marrow in reticulocytopenic cases, and the course of reticulocyte production indices with time and glucocorticoid treatment. The mean hematocrit at presentation was 24 mL/dL, but 30% of cases had an initial hematocrit <20 mL/dL. Median reticulocyte percentage at diagnosis was 9%, and median reticulocyte production index was 2.8 times basal. Twenty percent of cases had an initial reticulocyte count <4%, and 37% had an initial reticulocyte production index <2.0 times basal. These reticulocytopenic patients were nearly evenly distributed between warm and cold antibody-mediated cases and between primary and secondary cases. Fifty-four percent of reticulocytopenic cases had a bone marrow examination during hospitalization. Three-fourths of these marrows showed erythroid hyperplasia, and erythroid hypoplasia was seen in only one case. Eighty-eight cases

had serial reticulocyte measurements, and in only 15% of patients did the reticulocyte production index remain <2.0 times basal. Thus, in most cases, the initially low reticulocyte production index may represent a lag in marrow responsiveness to hemolytic stress. In cases with persistent reticulocytopenia, ineffective erythropoiesis is suggested by the frequency of marrow erythroid hyperplasia. In the cases that were initially reticulocytopenic and demonstrated an increase in reticulocyte production index, the magnitude of this increase was significantly greater in glucocorticoid-treated patients than in those not so treated, indicating that a glucocorticoid sensitive component exists in the marrow erythropoietic response to hemolysis. Awareness of the frequency of an initial reticulocytopenia in cases of autoimmune hemolysis may be important in initial diagnosis and treatment.

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AUTOIMMUNE hemolytic anemia has a wide spectrum of expression.^{1,2} Its onset can be abrupt and severe and can lead to a rapid and profound decrease in the hemoglobin concentration, which in elderly or chronically ill patients can result in death. In other subjects, RBC sensitization with antibody may not be associated with clinically evident hemolysis. This wide variability in the degree of shortening of RBC survival is further complicated by the variability in the response of the erythroid marrow to anemia. In most cases, a good reticulocyte response occurs and, in some cases, the combination of severe hemolysis and a brisk marrow response can lead to a situation in which all or nearly all of the erythrocytes in the blood are reticulocytes. The erythroid response, however, can be inappropriately low, and several reports or series²⁻²² have highlighted cases that have been associated with reticulocytopenia.

In these studies, we have defined more formally the erythropoietic response of patients with autoimmune hemolytic anemia to determine the frequency of reticulocytopenia, the state of the erythroid marrow in the reticulocytopenic patient, and the effects of time and glucocorticoid treatment on the reticulocyte response. Associated blood cell changes,

including the frequency of neutropenia and neutrophilia, of lymphopenia and lymphocytosis, and of thrombocytopenia and thrombocytosis are also presented.

MATERIALS AND METHODS

The medical records of 109 consecutive cases of autoimmune hemolysis at two University of Rochester hospitals (Strong Memorial Hospital and St Mary's Hospital) from 1965 to 1985 were reviewed. Charts were located by means of a computerized numerical diagnosis coding system. Criteria for inclusion as a case of autoimmune hemolysis were (a) a positive direct Coombs' test with anemia or clinical evidence for hemolysis or (b) a clinical diagnosis of autoimmune hemolytic anemia with elevated cold agglutinin titers in cases in which complete serologic test results were not available in the record (three cases). One case with a clinical diagnosis of Coombs' negative autoimmune hemolytic anemia was also included.

Age at diagnosis, year of presentation, sex, race, Coombs' test results, and hematocrit at presentation were recorded for all 109 patients. Initial reticulocyte counts were available in 108 cases. Other information recorded when available included the second, highest, lowest, and last reticulocyte counts during the hospital admission (88 patients), lowest and last hematocrit, first WBC count and WBC differential count, first platelet count, first mean corpuscular volume (MCV), first serum lactic dehydrogenase (LDH), and first total and direct bilirubin measurements. The description of the marrow aspirate and biopsy was recorded when available. Historical information obtained included associated illnesses, treatment administered, and number of transfusions. Cases were classified according to the following hemolysis subtypes: primary warm antibody, primary cold antibody, secondary warm antibody, secondary cold antibody, and drug-induced antibody.

Adjustments of the reticulocyte count for hematocrit (corrected reticulocyte count) and for determination of reticulocyte production index (RPI) were made.²³ Tests of statistical significance between group means or frequencies were done with Student's *t* test or chi-square test, respectively.²⁴ The microcoombs' test was performed using the antiglobulin consumption technique.²⁵ Serum LDH and bilirubin were determined using standard laboratory procedures. Indirect bilirubin was calculated as the difference between total and direct bilirubin measurements. Group data are expressed as the mean $\pm$ 1 SD unless otherwise specified.

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RESULTS

Patient Characteristics and Type of Hemolytic Antibody

One hundred-four of the 109 cases in this study were diagnosed while they were inpatients. Patients ranged in age from 4 months to 91 years. Seventy-one of the cases (65%) were female, and 38 were male (35%). Ninety-five percent of the patients were white and 5% were black.

Categories of hemolysis. Table 1 summarizes the frequency of hemolysis types identified by serologic and clinical studies. Nine of the drug-induced cases were associated with α -methyl dopa²⁶ and one with procainamide.²⁷ Three cases were not classifiable into one of these categories: One case had mixed features (warm and cold antibodies) and one case was direct Coombs' negative; in one case, the direct Coombs' test was positive but the γ and complement Coombs' reagent results were lost from the record. Four cases in this series had positive microcoombs' tests; ie, >40 molecules IgG per cell, when the direct Coombs' test was negative.

Presenting Hematocrits and Reticulocyte Counts

Hematocrit. At the time of diagnosis, the median hematocrit of patients was 24 mL/dL; range 9 to 56 mL/dL. The patient with the initial hematocrit of 56 mL/dL had severe chronic obstructive lung disease and cor pulmonale with higher baseline hematocrits. Figure 1 shows the cumulative frequency curve of presenting hematocrits.

Reticulocyte counts. The median reticulocyte percentage of patients at their presentation was 9% (range 0.4% to 92%). The mean reticulocyte count corrected to a hematocrit of 45 mL/dL (corrected reticulocyte count) was 7.4 ± 7.3 (median 5, range 0.1 to 45). The mean RPI was 3.8 ± 3.5 times basal (median 2.8, range 0.06 to 19 times basal). Figure 2 shows the cumulative frequency curve of the initial reticulocyte measurements. Reticulocyte counts were available in 108 of 109 patients. In the remaining case, the blood smear showed marked polychromatophilia, but this patient was excluded from analyses involving reticulocyte quantification. In the 88 cases in which serial reticulocyte counts were available, the first reticulocyte count was strongly correlated with the second reticulocyte count ($r = .87$, $P < .0001$). In 80% of cases with serial reticulocyte values available, the second reticulocyte count was obtained within 3 days of the first count.

By linear regression analysis, the reticulocyte percentage was significantly inversely correlated with the presenting

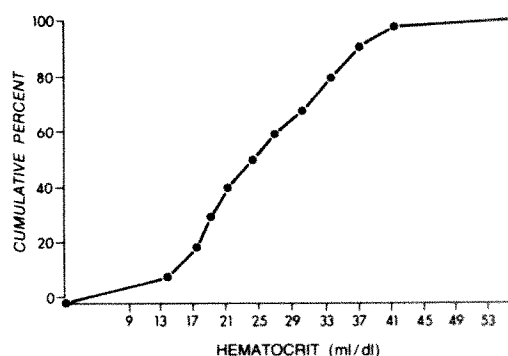


Fig 1. The cumulative percentage of hematocrits obtained at diagnosis in 109 cases. Median hematocrit was 24 mL/dL, and 30% of cases had an initial hematocrit of ≤ 20 mL/dL.

hematocrit ($r = -.35$, $P < .001$), although the correlation was weak. The corrected reticulocyte percentage and RPI did not correlate significantly with presenting hematocrit. Table 1 shows the mean presenting hematocrits and reticulocyte values based on the type of hemolysis. Although primary cold antibody-mediated hemolysis cases had a higher mean hematocrit and lower reticulocyte production index, the sample size was too small to reach conclusions regarding the significance of these differences.

Initial WBC and Platelet Counts

WBC counts were obtained in 108 patients. The mean WBC was $10,602 \pm 6,571$ cells/ μ L with a median of 9,000 cells/ μ L. The range was 1,500 to 137,000 cells/ μ L. Three WBC values $\geq 30,000$ cells/ μ L were excluded when the mean was calculated. Each of these three values occurred in a case of chronic lymphocytic leukemia. Median WBC counts were normal in both primary and secondary cases of warm hemolysis but were elevated in the primary and secondary cold antibody groups; 11,250 and 12,900 cells/ μ L, respectively.

Thirteen percent of cases had a decreased total WBC ($< 4,500$ cells/ μ L), and $\sim 40\%$ of all patients had an elevated total WBC ($> 11,000$ cells/ μ L). All 14 cases with leukopenia at diagnosis had warm antibody-mediated hemolysis and were equally divided between primary and secondary types. Mean hematocrit in the leukopenic group was 25 ± 8.6 mL/dL (median 23 mL/dL), and mean RPI was 2.9 ± 2.0 times basal (median 2.5). Six of the 14 cases with leukopenia

Table 1. First Hematocrit and Reticulocyte Count According to Type of Hemolysis

Hemolysis Type	Frequency of Cases (%)	(n)	Hematocrit (mL/dL)	Reticulocyte Count (%)	Reticulocyte Production Index ($\times$ Basal)
Primary warm antibody	47	51	$25 \pm 8.6(23)$	$18 \pm 20(10)$	$4.3 \pm 4.4(3)$
Secondary warm antibody	22	24	$25 \pm 9.4(24)$	$14 \pm 12(9)$	$3.4 \pm 2.1(3)$
Primary cold antibody	7	8	$32 \pm 11(30)$	$9.6 \pm 11(4)$	$3.0 \pm 2.8(2)$
Secondary cold antibody	12	13	$26 \pm 7.4(26)$	$11 \pm 7.1(8)$	$3.2 \pm 2.3(2)$
Drug-induced	9	10	$28 \pm 6.2(29)$	$13 \pm 10(10)$	$3.9 \pm 3.0(3)$
Unclassified	3	3	22	3.6	.85
All cases	100	109	$26 \pm 8.8(24)$	$15 \pm 16(9)$	$3.8 \pm 3.5(3)$

Data are presented as the mean $\pm$ SD. Median values are shown in parentheses. Medians and SD are not shown for the three unclassified cases.

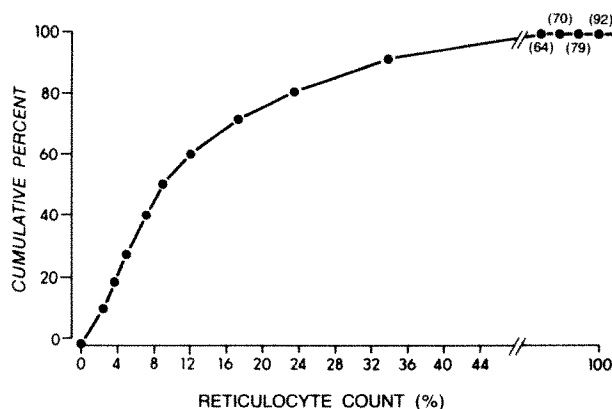


Fig 2. The cumulative percentage of first uncorrected reticulocyte percentage count obtained at diagnosis in 108 cases. Approximately 20% of patients had an initial reticulocyte count $<4\%$.

also had platelet counts $<120,000/\mu\text{L}$, and one other case developed thrombocytopenia later. Seven of the 14 had a palpable spleen, and one had splenic enlargement by radio-nuclide scan. None of the cases with leukopenia had received myelosuppressive drugs, and none had marrow infiltrative disease.

Of the patients with leukocytosis at presentation, approximately one-third had cold antibody-mediated hemolysis. Forty percent of these cases had initial reticulocyte production indices ≤ 2.0 times basal, but none had thrombocytopenia. Mean hematocrit in this group was $23 \pm 9.2 \text{ mL/dL}$. Figure 3A shows the cumulative frequency of the neutrophil count; Fig 3B shows the cumulative frequency of the lymphocyte count.

Initial platelet counts were available in 85 patients. An additional 19 patients had normal platelet estimates. Mean initial platelet counts did not differ significantly between subjects with warm antibody-induced or cold antibody-induced hemolysis. Figure 4 shows the cumulative frequency of initial platelet counts. Each of the 13 cases with thrombocytopenia at presentation was warm antibody-mediated, and these were divided approximately equally between primary and secondary types. Six of these cases had been given a clinical diagnosis of idiopathic thrombocytopenic purpura. One patient had chronic lymphocytic leukemia, and one had lymphoma with marrow involvement. Mean hematocrit in patients with thrombocytopenia was $30 \pm 7.6 \text{ mL/dL}$ (median 32), and mean RPI was 2.6 ± 1.5 times basal (median 2). In the 16 cases with thrombocytosis, 13 were warm antibody-mediated cases, two were secondary cold antibody-mediated cases in young adults, and one was a mixed warm-and cold-mediated case—also in a pediatric patient. Twelve of these 16 cases with thrombocytosis had a total WBC $>11,000 \text{ cells}/\mu\text{L}$. Mean hematocrit in those cases with thrombocytosis was $21.3 \pm 6.4 \text{ mL/dL}$ (median 21), and mean RPI was 3.9 ± 4.6 times basal (median 2.4).

Effect of Glucocorticoid Treatment on Reticulocyte Count

Serial reticulocyte counts were available in 88 cases. Glucocorticoids were used in 65 of these 88 cases (Table 2).

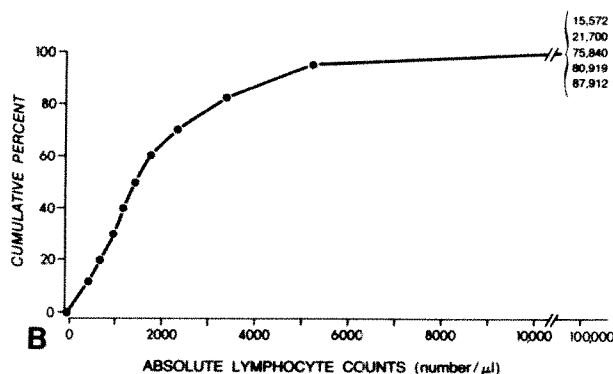
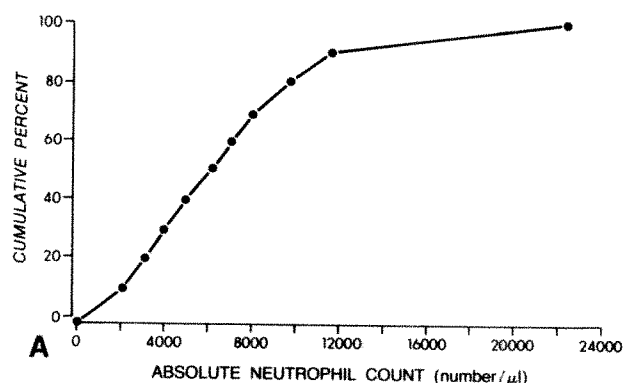


Fig 3. (A) Cumulative percentage of absolute neutrophil counts based on first WBC differential count obtained in 102 cases. Approximately 40% of cases had an absolute neutrophil count $>7,000/\mu\text{L}$. (B) Cumulative percentage of absolute lymphocyte count based on first WBC differential count in 102 cases. Eighteen patients had an absolute lymphocytosis $>4,000/\mu\text{L}$. Four patients had chronic lymphocytic leukemia, and five had cold antibody-mediated hemolysis.

Thirty-nine of these 65 cases had an increase in RPI while on steroids; in the other 26, the production index either remained stable or decreased. As summarized in Table 2, those who demonstrated an increase in reticulocyte production index while on steroids had lower mean initial hematocrits and RPIs than did those without such reticulocyte response. The magnitude of the increase in RPI in response

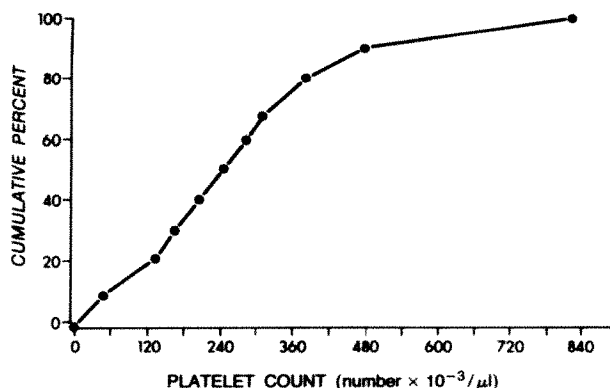


Fig 4. Cumulative percentage of initial platelet counts in 85 cases. Eighteen percent of patients had a platelet count $<120,000/\mu\text{L}$ and $\sim 20\%$ had a platelet count $>400,000/\mu\text{L}$.

Table 2. Effect of Glucocorticoid Treatment on RPI

Glucocorticoids	Effect on Reticulocyte Index	No. of Cases	Hct on Day of Initial RPI	Initial Reticulocyte Index	Highest Reticulocyte Index
Administered	Increase	39	22 ± 5.7	3.5 ± 2.9	7.2 ± 4.3
	No increase	26	26 ± 8.1	6.1 ± 5.2	—
None	Increase	14	23 ± 7.2	1.5 ± .93	3.8 ± 1.8
	No increase	9	30 ± 6.6	4.1 ± 5.6	—

RPI, reticulocyte production index; Hct, hematocrit.

Total cases = 88 with serial reticulocyte measurement available; data presented as mean ± SD.

to steroids (3.5 to 7.2 times basal) was significant as measured by the paired *t* test ($P < .01$). Seventy-five percent of cases reached their maximum RPI by 6 days after the initiation of glucocorticoid treatment, which represented the tenth day of hospitalization.

Fourteen of the 23 patients not treated with glucocorticoids also had an increase in their RPI. This mean increase (1.5 to 3.8 times basal) was also statistically significant ($P < .01$). Seventy-five percent of untreated cases attained their maximum increase in RPI by 10 days of hospitalization.

Analysis of Features in Cases Presenting With Low RPI

Figure 5 depicts the cumulative percentage graph of the initial RPI. Forty (37%) of the 108 cases with reticulocyte counts initially had an RPI of ≤ 2.0 times basal. About 30% of those with primary warm antibody-mediated disease, 30% with secondary warm antibody-mediated disease, 50% with primary cold antibody-mediated disease, 50% with secondary cold antibody-mediated disease, and 30% with drug-induced disease had an initial RPI ≤ 2.0 times basal. The higher percentage found in cases of cold antibody-mediated as compared with warm antibody-mediated hemolysis was not significantly different by chi-square test. A higher pro-

portion of the group with an RPI ≤ 2.0 was transfused (53% v 38%), and a lower proportion received steroids (65% v 87%), although these differences were not statistically significant by chi-square test. Mean hematocrit, platelet count, WBC, and serum LDH did not differ significantly between the two groups. The mean indirect bilirubin was significantly higher (3.3 ± 2 v 2.1 ± 1 mg/dL) in those with production indices ≤ 2 times basal, perhaps a reflection of both hemolysis and ineffective erythropoiesis.

As noted previously and in Table 2, 53 of 88 cases with serial reticulocyte values had an increase in RPI during hospitalization; in the other 35 cases, the index remained stable or decreased. As shown in Fig 6, 13 (15%) of the 88 cases had a maximum RPI that was ≤ 2.0 times basal, indicating persistent reticulocytopenia during hospitalization. Fifty-four percent of the 13 cases with persistently low reticulocyte values received steroids.

In 33 of 40 cases with initial RPI ≤ 2.0 , serial reticulocyte values were available. Twenty-five of the 33 or 76% had increases in RPI (1.2 to 4.1 times basal) ($P < .01$). In 15 of these patients who received glucocorticoids, this increase was more substantial (3.9 ± 3.6 times basal v 2.3 ± 2.0) than in those ten not receiving steroids ($P < .01$). Seventy-five

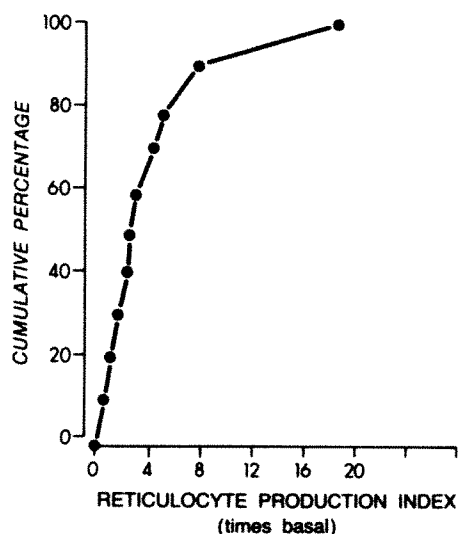


Fig 5. Cumulative percentage of 108 cases with a given reticulocyte production index (RPI) at presentation. Production index was calculated based on initial reticulocyte percentage and hematocrit values on the same day as described in the Materials and Methods section. Thirty-seven percent of cases had an initial production index ≤ 2.0 times basal.

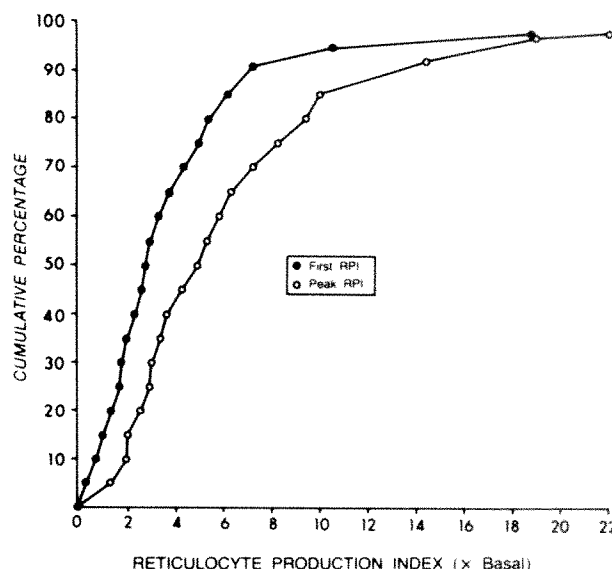


Fig 6. Comparison of cumulative percentage of the initial and peak reticulocyte production indices in the 88 patients in whom serial reticulocyte values were obtained. Although 37% of patients had a reticulocyte index ≤ 2.0 times basal initially, only 15% did not reach a reticulocyte index > 2.0 during serial observations.

percent of those receiving steroids had their highest RPI within 8 days of starting the hormone, which represented the twelfth hospital day. In the eight cases with an initial RPI ≤ 2.0 without a measured increase in reticulocyte production, 50% had received steroids.

Of the 55 cases with initial RPI > 2 and serial reticulocyte values, 28 (51%) had further increases in reticulocyte values during hospitalization. This increase was significant (4.6 v 8.1 times basal) as measured by paired *t* test, and all but one of these 28 patients were treated with steroids. Seventy-five percent of these patients had attained their highest RPI by the tenth hospital day, and in 75% this occurred within 5 days of starting steroid treatment. Twenty-two of the 27 cases with initial RPI > 2 who had no rise in RPI had received steroids.

A marrow aspirate or biopsy was performed in 21 of the 40 patients with RPI ≤ 2.0 (Table 3). Sixteen (76%) of these cases had erythroid hyperplasia. Only one patient had erythroid hypoplasia. Three patients had marrow involvement with lymphoma or leukemia.

Thirty-six of 68 cases diagnosed with an RPI > 2 had a marrow examination. Thirty-three (92%) had erythroid hyperplasia. In the other three cases, erythroid activity was not reported. One of these three cases showed marrow infiltration of small lymphocytes compatible with chronic lymphocytic leukemia, and another showed infiltration with breast carcinoma cells.

DISCUSSION

This series of cases of autoimmune hemolytic anemia shares many characteristics with prior reported series, including an ~70% incidence of warm antibody-mediated cases—excluding drug-induced cases,⁹ an age range extending from months to the tenth decade,^{2,9} and a case ratio of female to male of ~2:1.^{8,28} The spectrum of associated malignant and immunologic diseases is also similar to that found in prior series.^{2,9}

Although a wide range of both initial hematocrits and reticulocyte counts occurs in patients with autoimmune hemolysis, $>50\%$ of cases are diagnosed with hematocrit levels <25 mL/dL. The wide range of reticulocyte values at diagnosis and the weak inverse correlation of initial reticulocyte percentage to hematocrit confirm that the range and severity of anemia reflect several interacting variables, including the rate of hemolysis, the degree of marrow response to the hemolytic anemia, and the duration of the anemia.

The presentation of a significant proportion of patients with reticulocyte counts that do not suggest a marrow

response to hemolysis could lead to misdiagnosis of a hypoproliferative anemia or a missed diagnosis of immune hemolytic anemia if a Coombs' test is not obtained. Reticulocytopenia may contribute to the decision to transfuse or to withhold glucocorticoids more frequently in individual cases, a tendency that was noted in this series.

The subsequent increase in reticulocyte index in most patients who initially had reticulocytopenia is compatible with a more lengthy time dependency in their marrow response to hemolysis. The increase in reticulocyte index during hospitalization occurred as frequently in patients treated with glucocorticoids as in those not so treated. Pirofsky^{2,12} found that reticulocytosis usually occurred in patients with initially low reticulocyte counts with steroid therapy; however, no nontreated comparison group was reported in that series. In our study, glucocorticoid treatment did not seem to shorten the time to peak reticulocyte count in treated patients as compared with untreated patients. The difference in the peak reticulocytosis in glucocorticoid-treated patients as compared with untreated patients may indicate that the marrow response has a steroid-sensitive component as well. Glucocorticoid-treated patients had a peak reticulocyte index during serial study nearly twice that of untreated patients. A firm conclusion, however, cannot be drawn because this was not a prospective randomized study, and there may have been unidentified differences in the glucocorticoid-treated and untreated groups.

It is difficult to ascertain the prevalence of reticulocytopenia in autoimmune hemolysis from prior reports because patients in those studies were selected for type of hemolysis (warm v cold or primary v secondary), and reticulocyte production indices were not provided and reticulocyte values were not compared with hematocrit in individual cases. Allgood and Chaplin⁸ found that five of 47 idiopathic warm antibody-mediated cases had reticulocyte values $<5\%$. In their review, Crosby and Rappaport⁵ showed that 15 of 31 idiopathic cases were reticulocytopenic, as were 12 of 23 secondary cases. This series was based on case files from the Armed Forces Institute of Pathology, and the authors believed that the series selected for severe cases. Pirofsky² reported that 49% of reticulocyte counts were $<2\%$ in a group of 195 warm antibody-mediated cases. Pirofsky's series included cases with positive antiglobulin tests without documentation of hemolysis as well as cases of autoimmune hemolysis, and it contained a high proportion of secondary cases (82%). Twenty-six percent of idiopathic cases and ~50% of secondary cases had reticulocyte counts $<2\%$. In secondary cases, low counts were not related to chemotherapy or marrow infiltration.

Several mechanisms for reticulocytopenia in autoimmune

Table 3. Marrow Erythroid Cellularity in Patients With Higher and Lower Reticulocyte Counts

Reticulocyte Index	No. of Patients	Erythroid Hyperplasia	Erythroid Hypoplasia	Infiltrative Marrow Disease	Erythropoiesis Not Described
≤ 2.0	21	16(76%)	1	3	1
> 2.0	36	33(92%)	0	2	1

Fifty-seven of 108 patients with reticulocyte counts had a marrow examination. Of the 40 patients with a reticulocyte index ≤ 2 , 21 (53%) had a marrow examination. Of the 68 other patients who had a reticulocyte index > 2 , 36 (53%) had a marrow examination. The patient with erythroid hypoplasia had systemic lupus erythematosus.

hemolytic disease have been proposed. Some series have contained rare or infrequent cases with marrow aplasia, marrow infiltration, or megaloblastic changes.^{2,8} In a case of autoimmune hemolysis and marrow erythroid hypoplasia, an autoantibody distinct from the autoantibody against mature RBCs that was inhibitory to marrow erythroid colony-forming units (CFU-E) was demonstrated.²⁰ Some reports have described cases with marrow aplasia and prolonged reticulocytopenia,^{3,4,6,7,16,20} others described episodic RBC aplasia,¹⁵ and still others described transient marrow aplasia with reticulocytopenia, especially in childhood, possibly related to viral infections.^{10,11,22} Circumstances providing a clear-cut explanation for the poor erythroid response (ie, erythroid aplasia, tumor replacement of marrow, folate deficiency, or infection) are rare.

Marrow erythroid hyperplasia in reticulocytopenic cases of autoimmune hemolysis is also well described,^{2,5,13,17-19,21} and these cases comprise a far larger proportion of reticulocytopenic cases, as shown by our series. Cases of autoimmune hemolytic anemia, reticulocytopenia, erythroid cells in the marrow, but with inhibition of marrow erythroid colonies by patient plasma in vitro,¹⁹ and cases with retention of radioactive iron in the marrow and not in circulating RBCs²⁹ suggest that ineffective erythropoiesis can occur. The active erythroid marrow in the reticulocytopenic cases in our report is compatible with ineffective erythropoiesis.

The wide variability in initial WBC and platelet counts that occurs in autoimmune hemolytic anemia is also sugges-

tive of a wide range of marrow output response to the anemia and the "stress" state. Most cases of leukocytosis could not be explained by an underlying disease. The frequent coupling of leukocytosis and thrombocytosis in individual cases suggests a trilineage marrow production increase, even though in some of these cases, an appropriate reticulocyte response was not mounted. (Theoretically, this finding could be compatible with competition among lineages for stem cell differentiation commitments). The percentage of cases diagnosed with leukocytosis tended to be greater in patients with cold antibody-mediated hemolysis. Cases of leukopenia or thrombocytopenia occurred only in warm antibody-mediated cases, and underlying disease or splenomegaly did not usually account for these depressions of cell counts. The frequent coupling of leukopenia and thrombocytopenia is compatible with multiple lineage immune destruction in these cases, as first suggested by Evans and Duane in cases with prominent thrombocytopenia.³⁰

This series confirms and quantifies the wide range in the severity of anemia and in the marrow hematopoietic response to hemolysis in cases of autoimmune RBC destruction at the time of diagnosis. The significant proportion of cases diagnosed with reticulocytopenia has pathogenetic implications. Awareness of this occurrence is important to enhance prompt and accurate diagnosis and proper therapy.

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Multilineage Synergistic Activity Produced by a Murine Adherent Marrow Cell Line

By Peter Quesenberry, Zengxuan Song, Elizabeth McGrath, Ian McNiece, Richard Shadduck, Abdul Waheed, Gwen Baber, Ellen Kleeman, and Donald Kaiser

We reported previously that a cell line (TC-1) derived from adherent marrow cells produced colony-stimulating factor 1 (CSF-1) and a separate activity that acts synergistically with CSF-1 to stimulate giant macrophage colonies. We now report that an activity in TC-1 conditioned media (CM) separate from CSF-1 also synergizes multilineage colony formation by pure interleukin 3 (IL 3) and a crude source of granulocyte-macrophage colony-stimulating activity (GM-CSA) (murine lung-conditioned media). IL 3-induced megakaryocyte colony formation is also synergized. The CSF-1-dependent synergistic activity is not blocked by antibodies to IL 3 and is characterized as a nondialyzable (mol wt cutoff 3,000), heat-stable (56 °C, 30') activity that binds to DE-52 cellulose under conditions in which IL 3 does

not. This material has an apparent mol wt of ~200,000 by Sephadex G100 chromatography, and the bulk of it binds to Concanavalin A (Con A) and elutes off with α -methyl mannoside, indicating that it is a glycoprotein. As reported separately, these purified active fractions also have a pre-B cell-inducing activity. In addition, a non-IL 3 activity stimulates proliferation of the factor-dependent cell lines FDC-P1 and DA-1. These data indicate that an adherent marrow cell line produces a growth factor(s) that synergizes with IL 3, GM-CSA, and CSF-1 and induces pre-B cell formation. This may be an important regulator of early multilineage lymphohemopoiesis.

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PREVIOUSLY WE reported that a cell line (TC-1) isolated by differential trypsinization from the adherent cells present in long-term murine marrow liquid cultures produced colony stimulating factor 1 (CSF-1)¹ and a separate factor that did not have intrinsic colony-stimulating activity (CSA) but that synergized with CSF-1 to give rise to giant macrophage colonies.² Other researchers have reported on two separate activities that synergize with CSF-1: interleukin 3 (IL 3) and hemopoietin 1.³⁻⁵ IL 3 stimulates a wide range of colonies⁶ and thus differs from activity present in TC-1-conditioned media (CM). Hemopoietin-1, however, has no apparent intrinsic CSA and in this regard is similar to the TC-1 synergistic activity. In the present studies, we present biochemical, immunologic, and functional data indicating that the TC-1-derived synergistic activity is different from IL 3 or hemopoietin-1. In addition, we previously showed that crude CM (containing CSF-1) synergizes with IL 3 to stimulate giant mixed granulocyte-macrophage (GM)-megakaryocyte colonies. In this article, we extend those observations and demonstrate that the IL 3 synergy is not due to CSF-1. We show further that this activity potentiates a GM-CSA effect on bone marrow and stimulates the factor-dependent cell lines DA-1 and FDC-P1.⁷

MATERIALS AND METHODS

Cell Lines

TC-1 cell line. The TC-1 cell line, initially derived from C57B1/6J murine marrow,² was maintained in Fischer's media supplemented with 20% fetal calf serum (FCS), penicillin, and streptomycin. The cultures were incubated at 33 °C in 5% CO₂ and trypsinized (0.1%) and passaged every 4 to 7 days. CM were collected, centrifuged at 1,500 g, filtered through a 0.45- μ m Millipore membrane, and stored at -20 °C.

Factor-dependent cell lines. FDC-P1 cells⁷ (courtesy of Dr Jim Ihle, Frederick, MD) were grown in RPMI 1640 (GIBCO, Grand Island, NY) with 10% FCS and 25% CM from the WEHI cell line (courtesy of Dr Jim Ihle). The cells were incubated at 37 °C and 5% CO₂ in 25-cm² Corning flasks. Flasks were inoculated with 2×10^5 cells/mL and split 1:10 twice a week. DA-1 cells (courtesy of Dr Jim Ihle) were handled in a similar fashion.

Isolation of TC-1 CM-responsive FD cell lines. FDC-P1 cells were cloned in a soft-agar McCoy's clonal culture system (as used

for murine marrow colony-forming assays)⁸ (described later), in the presence of 10% to 20% 10 $\times$ TC-1 CM or 6% TC-1 CM partially purified by diethylaminoethanol (DEAE) chromatography. FDC-P1 colonies that formed after 14 and 24 days of growth were aspirated with a microliter pipette and dispersed in 35-mm Petri dishes containing 2 mL of RPMI 1640, 10% FCS, and 50% TC-1 CM. After 3 days, the cells were transferred to 60-mm Petri dishes (5 mL) and 3 days later were transferred to 25-cm² Corning flasks at 2×10^5 cells/mL. The cells were then split 1:5 to 1:10 twice weekly with reseeding kept at 2×10^5 cells/mL. After 2 weeks, cells were maintained in RPMI, 10% FCS, with 25% TC-1 CM.

Growth Factor Assays

Clonal agar assays. Marrow cells from tibias and femurs of normal ICR (Dominion Labs, Dublin, VA) or normal BDF₁ (Jackson Labs, Bar Harbor, ME) mice, aged 11 to 16 weeks, or BDF₁ mice given 150 mg/kg 5-fluorouracil (5-FU) intravenously (IV) 3 days prior to being killed were used as assay target cells. Alternatively, the factor-dependent cells FDC-P1 and DA-1 were used as assay cells in the same system. Growth factors were assayed using a modification of a double-layer agar technique⁹ or a single-layer technique.¹⁰ McCoy's media was supplemented with 0.8% minimal essential medium (MEM) essential amino acids, 0.4% MEM nonessential amino acids, 2 mmol/L of L-glutamine, 16 μ g/mL of L-asparagine, 8 μ g/mL of L-serine, 1 mmol/L of sodium pyruvate, 1×10^{-4} mol/L of 2-mercaptoethanol, and 15% pretested FCS. Marrow or factor-dependent cell line cells were included at different

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concentrations directly in the 0.3% agar-McCoy's layer. The substances to be assayed for growth factor activity were included in different concentrations in the 0.5% agar-McCoy's underlayer in most experiments with the double-layer systems; however, in some experiments using either single-layer or double-layer cultures, the stimulus was included in the 0.3% agar-McCoy's layer. These soft agar cultures (35-mm Petri dishes, Linbro 24-well plates, microtiter plates, or Terasaki dishes) were then incubated at 37 °C, 5% CO₂ for varying time intervals (1 to 22 days), dependent on the particular experimental protocol. IL 3 purified from WEHI-3-conditioned medium¹¹ was obtained from Dr James Ihle and CSF-1 purified from L cell CM¹² was obtained from Dr Richard Shadduck. Murine lung CM, prepared from ICR mice as previously reported,¹³ was used as a source of GM-CSF. Alternatively, CM from Concanavalin A (Con A)-stimulated Balb-C spleen cells was separated by DEAE-cellulose and Sephadex chromatography as described by Ihle and co-workers.¹⁴ Material separated from IL 3 and stimulating GM colony formation was used as a source of partially purified GM-CSF.¹⁴ The morphology of colonies was determined by microscopic analysis of whole agar mount slide preparations stained with either benzidine hematoxylin or acetylcholinesterase-hematoxylin as previously reported.⁸

Colony Criteria

Colonies of >50 cells were counted as granulocyte, macrophage, GM, or mixed megakaryocyte⁸ and colonies that contained three or more acetylcholinesterase-positive cells were scored as megakaryocyte colonies. Routinely, colonies were scored in agar dishes or whole agar mount slide preparations using an Olympus inverted microscope with an ocular micrometer as 0.5 to 1, or >1, 2, or 3 mm in greatest diameter, and as compact or loose. The loose category included all other groupings, some of which had relatively small numbers of cells widely scattered over a 2- to 3-mm diameter.

Mixed colonies were defined as colonies with at least 3 separate areas of the minor cell type or a single area of more than three cells. Almost all mixed colonies had >20 cells of the minor type, and most GM colonies of >1 mm had large numbers of each cell type. Other special colony types are cited in the Results section.

³HtdR Incorporation in Factor-Dependent Cell Lines

The effects of factors on FDC-P1 or DA-1 ³HtdR incorporation were assessed as described by Ihle and colleagues.¹⁴ The cells were washed three times with RPMI 1640 without WEHI-3 CM and resuspended in the same media at 1 × 10⁶ cells/mL. Doubling dilutions of test samples were added to 12 microtiter wells at 50-μL vol; 50 μL of cell suspension was then added to each microtiter well. Plates were then incubated at 37 °C, 5% CO₂ for 24 to 72 hours and pulsed with 1 μCi/well of ³HtdR (specific activity 6.7 Ci/mmol) 5 hours prior to harvest. Cells were harvested on a Skatron harvester; ³HtdR incorporation was then determined by counting on a Beckman Beta-scintillation counter. Results are expressed as cpm.

CSF-1 Studies and Immunoabsorption

CSF-1 levels were determined by radioimmunoassay (RIA), and antiserum to CSF-1 was produced in male New Zealand white rabbits as previously reported.¹⁵ This antiserum was covalently bound to cyanogen bromide-activated Sepharose 4B. TC-1 CM (usually 10× concentrated) was applied to this column after preparatory dialysis against 0.05 mol/L of Tris, 0.15 mol/L of NaCl, and 0.3% polyethylene glycol (PEG) buffers, pH 7.5, to remove CSF-1. The run-through or unbound material was collected, the column was rinsed with 300 mL of buffer, and the CSF-1 was eluted with 25 mL of 2 mol/L of guanidine pH 4.0 in starting buffers. Guanidine was

removed from the purified CSF by repeated diafiltration in an Amicon ultrafiltration cell.

Biochemical Characterization of CSF-1-Dependent Synergistic Activity

Collection starting material. Initial studies were made using TC-1 CM with 5% FCS. With Nutridoma-SF supplement, the FCS concentration was reduced to 1%, and this material was used for some of the more recent studies. Thus far, reduction to no serum results in viable cells that do not produce synergistic activity. Material was pooled, and stored at -20 °C until further manipulation.

Concentration and protein determination. Amicon ultrafiltration units or a spiral cartridge was used with YM 10 (10-kd mol wt cutoff) or YM 100 (100-kd mol wt cutoff) ultrafiltration membranes. No significant loss of bioactivity occurred in the concentrates. Total protein was determined by the Bradford assay.¹⁶

Ammonium sulfate precipitation. Ammonium sulfate precipitations were performed by addition of solid crystal ammonium sulfate (Ultrapur—Schwartz/Mann Biotech, Cambridge, MA). An initial cut of 0% to 35% (0.209 g/mL) was done by slow addition of crystal to a stirred flask of media. The sample was precipitated for 20 minutes at 4 °C. The mixture was centrifuged at 10,000 rpm in an IEC B20A centrifuge in 200-mL vol for 30 minutes at 4 °C. The supernatant was decanted, and a second cut of 35% to 65% (0.198 g/mL) was carried out. The precipitate from the second cut was resuspended in a minimal volume of 0.01 mol/L Tris-HCl pH 7.4, 0.02% Na azide, 0.01% PEG 6000, and dialyzed in the same buffer for 1 to 2 days at 4 °C with three changes per day.

DEAE-Cellulose chromatography. DE-52 resin (Whatman, Clifton, NJ) was prepared, defined in a standard manner, and then equilibrated in 0.01 mol/L of Tris-HCl pH 7.4. TC-1 CM 1× (4 to 5 liters) was added to the resin at a TC-1 CM/resin volume ratio of 4:1 or 5:1, stirred gently, and placed at 4 °C for 2 hours with gentle stirring every 30 minutes. The slurry was poured through a Kimex funnel, and the nonadherent fraction was set aside. The resin was then washed with 0.01 mol/L of Tris HCl pH 7.4 until the wash was free of protein. The adherent proteins were eluted from the resin with 1 mol/L of NaCl in 0.01 mol/L of Tris HCl pH 7.4 or with a linear gradient of 0 to 0.8 mol/L of NaCl. The eluate was concentrated using an Amicon spiral cartridge and 10K membrane to ~100 mL, and this concentrate was dialyzed against 0.01 mol/L of Tris HCl pH 7.4 for 1 to 2 days with three changes per day.

Sephacryl S300 chromatography. A Sephacryl S-300 column (2.5 cm × 49.5 cm, 243-mL bed volume) was equilibrated to 0.5 mol/L of NaCl, 0.02 mol/L of Tris HCl pH 7.4, 0.02% Na azide, and 0.01% PEG-6000. Fractions of 6 mL were collected at 70 mL/h. Samples to be applied were dialyzed in the equilibrating buffer. For bioassays, fractions were pooled in groups of five and concentrated on Amicon 10K membrane threefold.

Sephadex G100 chromatography. Sephadex G100 (Superfine, Pharmacia, Piscataway, NJ) (2.5 × 85 cm/resin 417-mL vol) was equilibrated with 2 L of 0.01 mol/L of Tris-HCl pH 7.4, 0.02% Na azide, and 0.01% PEG 1000; the sample was applied (void vol 1% to 20%) and run at 60 mL/h. Fractions of 15 to 18 mL were collected and pooled (three to five fractions per pool), concentrated (three to five times), and sterile-filtered (0.22 μ) for bioassay.

Con A Sepharose chromatography. Con A Sepharose (Pharmacia) was equilibrated in 0.5 mol/L of NaCl, 0.02 mol/L of Tris-HCl pH 7.4, 0.01% PEG-6000, 0.02% Na azide, and a column (2.5 × 14.5 cm) with a bed volume of 72 mL generally used. Samples of TC-1 applied generally were active fractions that had been sequentially separated by DE-52 and/or Sephadex G-100 or Sephacryl S-300 chromatography. The sample, equilibrated in the

Table 1. Effect of Anti-IL-3 Antibody on TC-1 CM Stimulated Giant Colony (>1 mm) Formation

Group	Giant Colony/10 ⁶ Cells
TC-1-10× (8.3%) + McCoy's 1× (8.3%)	20 ± 3
TC-1-10× (8.3%) + anti-IL 3 IgG (1 mg/mL)	23 ± 2.4
TC-1-10× (8.3%) + control IgG (1 mg/mL)*	29.3 ± 2.4

CM, conditioned media.

In this experiment, anti-IL 3 antibody blocked colony formation by 166 U/mL of IL 3; in other experiments, a level of 1 mg/mL was sufficient to block FDC-P1 proliferation completely.

*IgG purified from normal rabbit sera by protein A chromatography (courtesy of Dr James Ihle).

Con A equilibration buffer was applied, the run-through was collected, the column was washed with 100 to 150 mL of the equilibration buffer, and the column was then eluted with 500 mL of an α -methyl mannoside gradient (0 to 0.25 mol/L). Fractions of 4 to 5 mL were collected at 10 mL/h, pooled (five fractions per pool), concentrated on an Amicon 10K membrane, and dialyzed into phosphate-buffered saline (PBS) pH 7.4 for subsequent bioassay.

RESULTS

Previously, we reported that the murine marrow adherent cell line TC-1 produced CSF-1 and a factor that interacted synergistically with CSF-1 to stimulate giant macrophage colonies. This factor had no intrinsic colony-forming ability, but may still represent relatively low levels of IL 3. Accordingly, we evaluated the capacity of two different antibodies to IL 3 to block giant colony formation induced by TC-1 CM. IL 3 antibodies at a concentration that abolished IL 3 colony formation and FDC-P1 (the IL 3-responsive assay cell line) proliferation did not block giant macrophage colony formation (Table 1). These data suggest that the TC-1 synergistic activity is not IL 3.

Synergism With IL 3

IL 3 has been shown to stimulate granulocyte, macrophage, GM-megakaryocyte and mixed megakaryocyte-GM colony formation. We previously established half-maximal values for IL 3-stimulated hemopoietic colony formation; depending on colony type, these ranged from 0.14 to 0.76 ng/mL.⁶ Plateau levels for all types of colony formation were

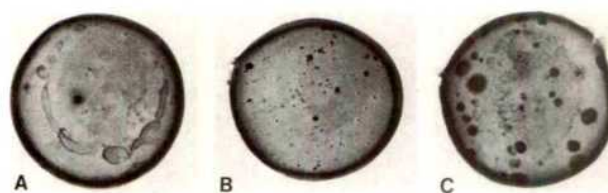


Fig 1. Synergistic effect of TC-1 conditioned media (CM) on Interleukin 3 (IL 3) stimulation of post-5-fluorouacil (5-FU) marrow cells. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin (magnification $\times 1.3$). Murine BDF₁ marrow cells were harvested 3 days after 150 mg 5-FU/kg body weight and 0.5×10^5 cells/mL were cultured in a double-layer agar assay with 1.7 ng/mL of IL 3 (panel A), 8.3% 20 $\times$ TC-1 CM (panel B) or 1.7 ng/mL IL 3 plus 8.3% 20 $\times$ TC-1 CM (panel C). Slide preparations were made after 22 days of culture.

achieved with concentrations of >3 ng/mL and, using these plateau levels of IL 3, we previously showed that crude TC-1 CM (with CSF-1) interacts with IL 3 to induce giant mixed GM and GM-megakaryocyte colonies from normal murine marrow.¹⁷ In this setting, total colony formation is increased only modestly by the combination of TC-1 CM and IL 3, with most of the increase accounted for by GM colonies (Table 2). The major synergistic effect is thus on the size and cell content rather than on the number of colonies. Furthermore, only with the IL 3/TC-1 CM combination were giant compact colonies of granulocytes, macrophages, and megakaryocytes seen. In the present studies, we show a similar potentiating effect of TC-1 CM plus IL 3 on target marrow harvested at 1 and 3 days after 5-FU 150 mg/kg, but peak growth and maximum effect occurred somewhat later than with normal marrow, ranging from 14 to 24 days of growth in soft agar culture (Fig 1). Potentiation of IL 3-induced colony formation by TC-1 CM occurred at from 0.52 to 16.7 ng IL 3/mL and was noted at different TC-1 CM plateau levels (data not shown). Because CSF-1 acts synergistically with IL 3 to give rise to giant macrophage colonies and CSF-1 is present in the crude TC-1 CM, it was important to study the role CSF-1 might play in the observed synergism between TC-1 CM and IL 3. Accordingly, we studied TC-1 CM that had been totally depleted of CSF-1 (as determined by RIA) by affinity CSF-1 antibody column chromatography. Three different pools of TC-1 CM (concentrated ten times) were passed over a Sepharose column to which

Table 2. Colony Formation (>50 Cells) Stimulated by Different Hematopoietic Growth Factors

Colony	Colony/0.5 $\times 10^5$ Marrow Cells				
	TC-1	IL 3	LCM	TC-1 + LCM	TC-1 + IL 3
Macro	109 ± 10	72 ± 9.7	28.7 ± 4.6	33.1 ± 4.7	92.3 ± 11.5
GM	26.1 ± 4.7	26.5 ± 6.8	50 ± 5.6	71.1 ± 5.3	57.7 ± 4.2
Gran	16.4 ± 3.2	31.8 ± 7.9	25 ± 6.5	20.3 ± 1.6	24.2 ± 3
Mega	0	8.8 ± 3	2 ± 0.6	1.25 ± 0.8	5.9 ± 2.3
Total	151 ± 11.4	139 ± 10.2	106 ± 6.8	126 ± 7.8	180 ± 9.8

IL 3, Interleukin 3; Macro, macrophage; GM, granulocyte-macrophage; Gran, granulocyte; Mega, all megakaryocyte colonies of over 3 cells plus mixed megakaryocyte colonies; total, sum of all colony types.

Data are presented as mean $\pm$ 1 SEM from 8 to 9 individual slide preparations from 3 separate experiments. Stimuli either by themselves or in combination were tested at the following concentrations: TC-1 conditioned media (CM) 8.3% to 15.4%; IL 3 7.7 to 16.7 ng/mL; and LCM 7.7% to 8.3%.

Table 3. Effect of TC-1 "Unbound" Fraction on IL-3-Induced Colony Formation

Group	Mega	Colonies/5 × 10 ⁴ Cells		
		Total Mega	>2 mm	>1 mm
IL 3	7.5 ± 4.3	10 ± 4	3.7 ± 1.2	51 ± 19
IL 3 + unbound fraction	17.3 ± 4.7	24.3 ± 6.2	19 ± 9.3	67 ± 21

These data are from three separate experiments testing TC-1 conditioned media (CM) that had been passed over an anti-CSF-1 Sepharose column (unbound fraction) and was free of CSF-1 activity by radioimmunoassay. In these experiments, both normal murine ICR and BDF₁ target marrow cells were used, and Interleukin 3 (IL 3) levels varied between 43 and 160 U/mL. Unbound activity represented the run-through fraction and thus was equivalent to a 10× TC-1 CM concentrate devoid of CSF-1. Potentiation occurred in different experiments with final unbound concentrations of 1% to 25%. Unbound fractions at higher concentrations ordinarily caused inhibition of growth but this inhibition varied with different pools, and the concentration at which it was seen varied considerably between the different pools.

Mega, pure megakaryocyte colonies of more than three cells; total mega, pure and mixed mega colonies; >2 mm mixed, colonies >2 mm in diameter predominantly granulocyte-macrophage (GM) or macrophage, but also including GM-Mega.

purified anti-CSF-1 was covalently bound. The run-through or unbound fraction had no CSF-1 by RIA and did not stimulate murine marrow colony formation. The CSF-1 eluted off the column with guanidine synergized with the unbound material to give giant colonies, confirming our previous observations.² This unbound material when added at different concentrations to different levels of IL 3 (five separate experiments) potentiated megakaryocyte colony formation and the formation of giant GM and GM-megakaryocyte colonies (Table 3). The actual numbers of different-sized groups fail to convey the true extent of this synergy, as illustrated in two experiments assessing synergy with normal or post-5-FU marrow (Figs 2 and 3). Colonies characteristic of IL 3 plus TC-1 stimulation were very large with a tight central core of cells consisting of granulocytes, macrophages, and megakaryocytes. Furthermore, with post-5-FU marrow colonies of >2 to 3 mm in diameter with central dense cells of >1 mm were seen only in the TC-1 unbound material + IL 3 group (7 ± 1/5 × 10⁴ cells), not in the IL 3 group.

The crude TC-1 CM plus IL 3 tested against post-5-FU marrow over 14 to 22 days of clonal agar culture had variable

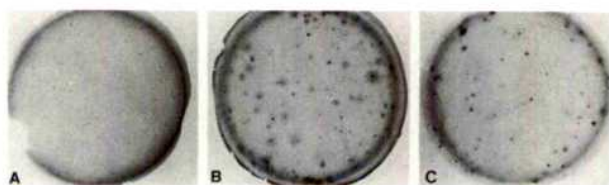


Fig 2. Synergistic effect of TC-1 "unbound" fraction on Interleukin 3 (IL 3) stimulated colony formation. Photomicrograph of whole agar slide preparations stained with acetylcholinesterase and hematoxylin. Murine BDF₁ cells (0.5 × 10⁶/mL) were cultured in a single-layer agar assay with 25% unbound fraction (panel A), 25 ng/mL of IL 3 plus 25% unbound fraction (panel B), or 25 ng/mL of IL 3 (panel C). Slide preparations were made after 7 days of culture.

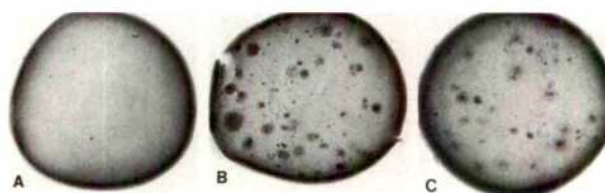


Fig 3. Synergistic effect of TC-1 "unbound" fraction on Interleukin 3 (IL 3) stimulation of post-5-fluorouracil (5-FU) marrow cells. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin. Murine BDF₁ marrow cells were harvested 3 days after 150 mg of 5-FU/kg body weight and 0.5 × 10⁶ cells were cultured in a double-layer agar assay with 2% unbound fraction (panel A), 2% unbound fraction plus 4.3 ng/mL of IL-3 (panel B), or 4.3 ng/mL of IL 3 (panel C). Slide preparations were made after 22 days of culture.

effects on the numbers of colonies >50 cells. The addition of TC-1 CM to IL 3 resulted in total colony formation that ranged from 56% to 95% of the combined IL 3 and TC-1 CM controls (ie, adding numbers of colonies stimulated by IL 3 alone and TC-1 CM alone). When the "unbound" TC-1 material was added to IL 3 and tested against post-5-FU marrow, total colony (>50 cells) formation was reduced to 74% to 85% of the IL 3 control. Thus, the major effect of the synergistic activity in TC-1 CM when added to IL 3 is to increase colony size with no overall stimulation (and in some cases, apparent inhibition) of total colony formation.

Similar results occurred when plateau concentrations of murine lung CM (a source of GM-CSA-2) were assayed in the presence of plateau concentrations of TC-1 CM; giant mixed GM colonies predominantly of the loose type were seen (Fig 4). Again, within a specific size range and category, colonies from the TC-1 + LCM group had more cells. Total colony formation was modestly depressed by the TC-1 + LCM combination as compared with TC-1 alone, but individual GM colony formation was augmented (Table 2). Again this synergizing effect was seen over varying concentrations of 10× TC-1 CM (0.4% to 11.5%), LCM (0.4% to 11.5%), or 10× LCM (1.19% to 3.8%) and when post-5-FU marrow was used as a target marrow (Fig 5). In the latter case, as with the IL 3 studies, peak growth and potentiation effect with the 5-FU marrow occurred at 14 to 22 days of soft agar culture. The effect of TC-1 CM on LCM-induced colony formation was confirmed in six separate experiments.

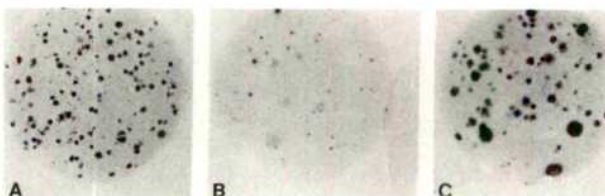


Fig 4. Synergistic effect of TC-1 conditioned media (CM) on LCM-stimulated colony formation. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin (magnification ×1.3). Murine BDF₁ marrow cells (0.5 × 10⁶ cells/mL) were cultured in a double-layer agar assay with 11.5% 10× TC-1 CM (panel A), 3.8% LCM (panel B), or 11.5% 10× TC-1 CM plus 3.8% LCM (panel C). Slide preparations were made after 14 days of culture.

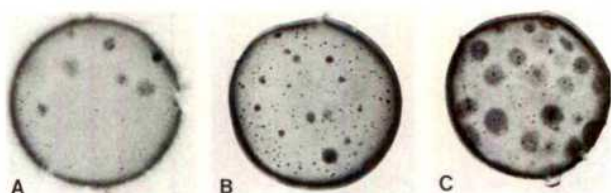


Fig 5. Synergistic effect of TC-1 conditioned media (CM) on LCM stimulation of post-5-fluorouracil (5-FU) marrow cells. Photomicrograph of whole agar (double-layer) slide preparations stained with acetylcholinesterase and hematoxylin (magnification $\times 1.3$). Murine BDF₁ marrow cells harvested 3 days after 150 mg of 5-FU/kg body weight were plated at 0.5×10^6 cells/mL in double-layer agar and cultured for 22 days with 8.3% LCM (panel A), 8.3% 10 $\times$ TC-1 CM (panel B), or 8.3% LCM plus 8.3% 10 $\times$ TC-1 CM (panel C).

We also evaluated unbound fractions free of CSF-1 for their ability to synergize with murine lung-CM, a crude source of GM-CSA, or with a partially purified GM-CSA derived from Con A-activated Balb-c spleen CM (three separate experiments). The potentiation, although less dramatic than that which occurred with unseparated TC-1 CM, was still present (Fig 6) affecting predominantly GM and M colonies. As with unbound material interacting with IL 3, variable high-dose inhibitors were noted.

Characterization of the CSF-1-Dependent Synergistic Activity Present in TC-1 CM

Using giant macrophage colony formation (>0.5 - and >1.0 -mm diameter dense colonies) and carrying out clonal agar cultures with and without added pure CSF-1 (400 to 800 U/mL), we characterized the CSF-1-dependent synergistic activity present in TC-1 CM as nondialyzable (membrane cutoff 3,000), relatively heat stable (56 °C for 30 minutes) with a pH range between 4 and 8. It is stable on storage at 4 °C for at least 6 weeks and is stable to three or four freeze-thaws. During purification, synergistic activity was seen in fractions that also had significant levels of CSF-1, and adding CSF-1 in the assays did not reveal fractions with SA separate from CSF-1. Thus, the data presented represent the capacity of the various separated fractions to stimulate giant colony formation without added CSF-1. The SA was retained on both Amicon PM 10 (10,000 mol-wt cutoff) and Amicon PM 100 (100,000 mol-wt cutoff) and precipitated between 35% and 65% ammonium sulfate. The synergistic activity bound to DE-52 cellulose (What-

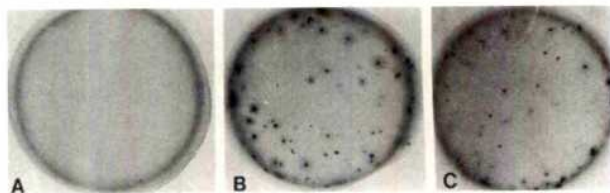


Fig 6. Synergistic effect of TC-1 unbound fraction on LCM-stimulated colony formation. Photomicrograph of whole agar slide preparations stained with acetylcholinesterase and hematoxylin. Murine BDF₁ cells (0.5×10^6 /mL) were cultured in a single-layer assay with 12.5% unbound fraction (panel A), 12.5% unbound fraction plus 12.5% LCM (panel B), or 12.5% LCM (panel C). Slide preparations were made after 7 days.

man, Clifton, NJ) that had been equilibrated in 0.02 mol/L of Tris-HCl, pH 7.4 (conditions in which IL 3 does not bind) and eluted from the column between 0.1 and 0.4 to 0.5 mol/L of NaCl (Fig 7). TC-1 CM after ammonium sulfate precipitation or DE-52 cellulose batch elution and separation by Sephadex G100 or Sephacryl S300 chromatography had an apparent mol wt of $>60,000$ ($\sim 200,000$ by Sephadex G100) (Fig 8). The bulk of SA from crude or partially purified TC-1 CM bound to Con A eluting with α -methyl mannoside (Fig 9). A series of sequential separations resulted in significant purification and increase in specific activity (Table 4).

TC-1 CM was separated by DE-52 cellulose chromatography followed by G100 Sephadex chromatography. Active pools from the G100 column were rendered CSF-1-free by passage over an affinity CSF-1 antibody column. This "unbound" material synergized with stage-1 CSF-1 to give tight colonies >0.5 mm in diameter (Fig 10). The same unbound material tested at 13% concentration against normal murine marrow in the presence of IL 3 increased large (>1 mm) colony formation to 169% of the IL 3 control and, at a 5% concentration, caused a 3.14-fold increase in murine pre-B cell generation (as determined by cytoplasmic IgM-positive cells) in a liquid culture system.¹⁸ In addition, when target bone marrow cells from Balb/c mice harvested 8 days after 150 mg of 5-FU/kg were used and these cells were cultured for 14 days at 7% O₂, this same unbound preparation tested at 13% augmented IL 3 stimulation of colonies (>0.5 mm) by 360% of control. Similarly, material that had been subjected to sequential separation over DE-52 cellulose, G100 Sephadex and Con A chromatography (the α -methylmannoside eluate) and that had been rendered free of CSF-1 by CSF-1 antibody affinity chromatography increased pre-B cell generation fivefold.¹⁸

Other Growth-Promoting Activities in TC-1 CM

We recently reported the presence of a murine pre-B cell-inducing activity in crude and partially purified TC-1 CM separate from CSF-1.¹⁸ This activity was found in synergistic activity-active fractions separated by sequential

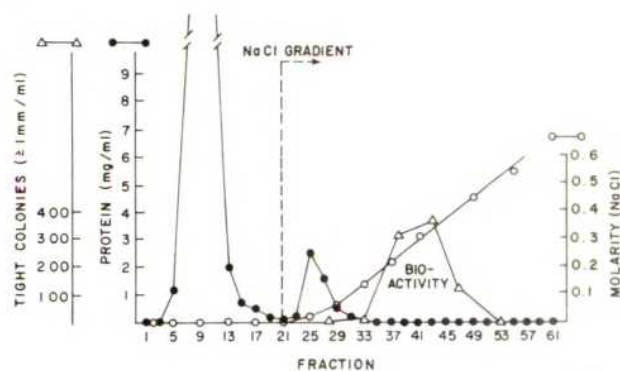


Fig 7. Separation of TC-1 conditioned media (CM) on DE-52 columns. TC-1 CM (5% fetal calf serum (FCS), concentrated 10 $\times$ Amicon PM10), ammonium sulfate precipitated (35% to 65%), dialyzed, and applied to DE-52 column in 0.02mol/L of Tris-HCl pH 7.4. NaCl gradient (0 to 0.8 mol/L) buffer was applied. Bioactivity is defined as >1 mm macrophage colonies per milliliter with double-layer agar system using normal ICR mouse bone marrow.

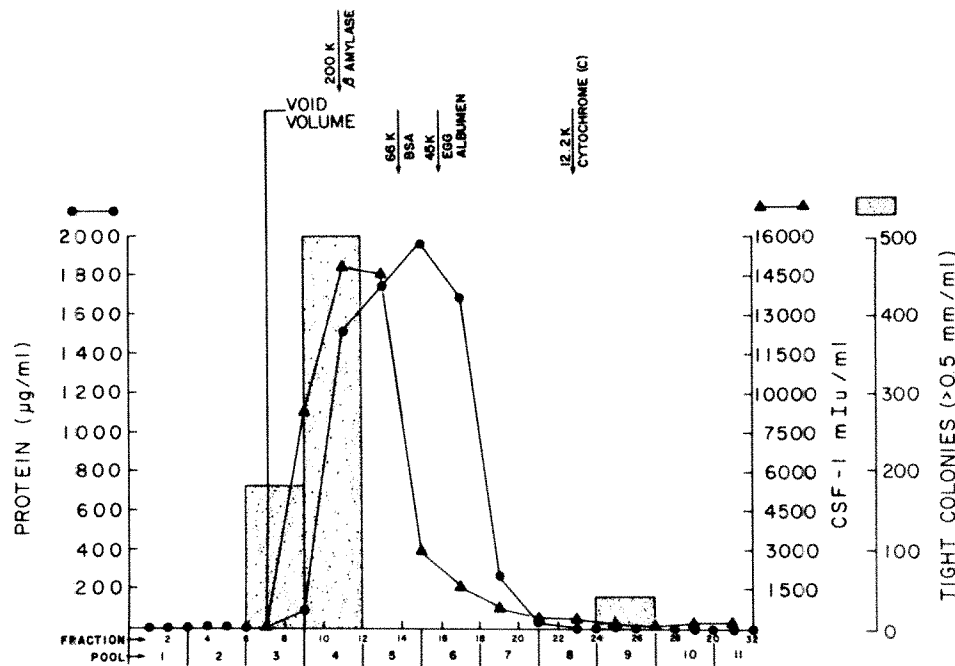


Fig 8. Separation of TC-1 conditioned media (CM) by Sephadex G100 chromatography. Two 7-L pools of TC-1 CM (5% fetal calf serum) were separated by batch diethylaminoethanol (DEAE)-cellulose chromatography, and the active salt eluates were then equilibrated with 0.01 mol/L of Tris HCl pH 7.4, 0.02% Na azide, and 0.01% polyethylene glycol (PEG)-6000. Fifty milliliters of this eluate was applied to a Sephadex G 100 (2.5 × 85 cm/resin volume = 417 mL) column that had been equilibrated with 2 L of 0.01 mol/L of Tris HCl pH 7.4, 0.02% Na azide, and 0.01% PEG-6000. Fractions were collected at a rate of 60 mL/h with 16 mL per fraction, and aliquots from three fractions were combined for each pool, concentrated (1.8 to 3 times), and sterile filtered (0.22 U/mL). CSF-1 on the pools was determined by radioimmunoassay (RIA),¹⁶ and protein was determined by Bradford assay.¹⁸ Growth factor results are expressed as the number of tight colonies of >0.5-mm diameter stimulated per milliliter of CM.

DE-52 cellulose, Sephadex G100, Con A, and CSF-1 antibody affinity chromatography. We also noted an activity in TC-1 CM stimulating FDC-P1 (Fig 11) or DA-1 cell proliferation. This material also stimulated FDC-P1 cells that had been selected for TC-1 growth factor sensitivity, although the kinetics of stimulation were altered; moreover, in this case, by 74 hours of growth, there was a higher rate of proliferation of the FDC-P1 TC-1 sensitive cells grown in TC-1 CM as compared to WEHI-3 CM (a source of IL 3) (Fig 11).

DISCUSSION

We present a characterization of a multilineage synergistic activity produced by the murine adherent marrow cell line TC-1. We have shown that a non-IL 3 activity is capable of synergizing with IL 3 to produce giant mixed GM and GM-megakaryocyte colonies and to enhance megakaryocyte colony formation. These data show that CM from the cell line devoid of CSF-1, and with no intrinsic colony-forming ability by itself, contains this synergizing activity for IL 3. This synergy occurs against both normal bone marrow and marrow from animals pretreated with 5-FU (Figs 2 and 3). Similarly, an activity present in the TC-1 CM is capable of synergizing with a source of GM-CSA, ie, murine lung CM, to give rise to giant GM colonies (Figs 4 through 6). This effect, while lessened with the removal of CSF-1, is still present. We previously showed the existence of an activity that synergizes with CSF-1¹²; thus, the TC-1 cell line is

capable of producing a bioactivity that has no intrinsic CSA but that appears to be able to augment the effects of three separate regulators—CSF-1, IL 3, and probably GM-CSA. In these same preparations, we also found an activity capable of inducing pre-B cells in short-term cultures of murine marrow cells.¹⁸ Thus, we found activities from the TC-1 stroma line that appear to act early in both B lymphopoiesis and myelopoiesis.

Pre-B lymphocyte-inducing factor was first found in the urine of patients with cyclical hematopoiesis.^{19,20} In this condition, at a time when myelopoiesis is depressed and just preceding a wave of pre-B cells in the bone marrow, high levels of a pre-B-inducing activity exists in the urine of these patients.²⁰ We may be dealing with a similar type of activity from the adherent cell line described in this article. This cell line was isolated from long-term murine cultures by differential trypsinization using Fischer's media supplemented with FCS and no hydrocortisone. This was initially done to facilitate isolation but we now realize that these conditions may well be permissive for B lymphopoiesis in vitro and approach those described by Whitlock-Witte for long-term B lymphocyte cultures.²¹ In addition, Dorshkind recently showed that the classical myeloid Dexter murine cultures can be switched to cultures supporting B lymphopoiesis by altering the culture conditions. These alterations included using horse serum rather than FCS and omitting hydrocortisone.²² This suggests that one primary stromal cell may be able to provide the environment for supporting both B lymphopoiesis

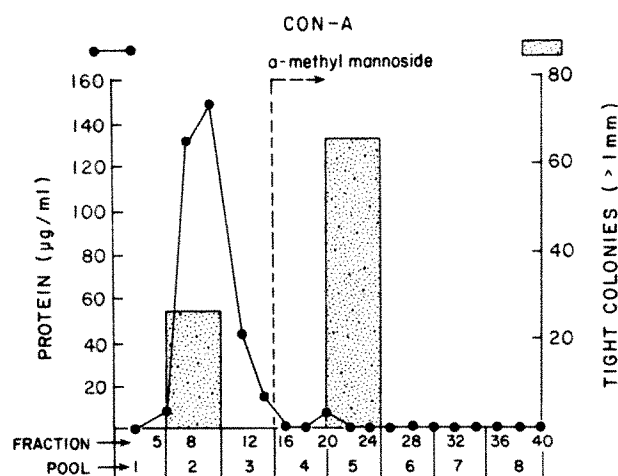


Fig 9. Separation of TC-1 conditioned media (CM) by Concanavalin A (Con A) Sepharose affinity chromatography. TC-1 CM eluted from batch DE-52 resin was applied to Con A Sepharose column and eluted in 0.1% α -Methylmannoside in 0.5 mol/L of NaCl, 0.02 mol/L of Tris-HCl pH 7.4, 0.02% azide, 0.001% polyethylene glycol (PEG)-6000. HPP/mL defined as >1-mm macrophage colonies in double-layer agar system using normal ICR mouse bone marrow.

and myelopoiesis and that the lineage favored may be dependent on the specific conditions of the cell. It is possible that the myeloid synergizing activity and the pre-B factor seen in the TC-1 supernatants represent the same factor (thus far, they co-purify in biochemical purifications) and that this factor is produced by stromal cells under either condition. The final pathway may be determined by the production of additional factors specific for B lymphopoiesis or myelopoiesis. In support of this hypothesis, the Dexter culture system appears to produce both CSF-1² and GM-CSA,²³ thus providing the terminal differentiating hormones that could account for all the noted phenotypic end cells produced in this system, ie, granulocytes, macrophages, and megakaryocytes. In the CM from the TC-1 line, we recently found an activity similar to that of BCGF-2 which stimulates differentiated B cell proliferation.²⁴ The activity in the TC-1 conditioned media is unique in that IgM secretion is not stimulated. This suggests the presence of a differentiated B cell factor along with an earlier acting B cell factor, possibly providing a model of factor relationships in the Whitlock-

Table 4. Purification of TC-1 Derived LHGF

Sample	Tight Colony/ 5×10^4 Cells/mg Protein		Total Units	
	>0.5 cm	>1 cm	>.5	>1
Original TC-1 CM*	59	31	544,864	289,249
DEAE-salt eluate	3,487	1,601	586,925	269,473
G-100 Sephadex Pool 3	7,996	3,553	366,537	162,870

Abbreviation: LHGF, lymphohemopoietic growth factor.

*Starting pool of 7 L of 5% TC-1. Only 50% of diethylaminoethanol (DEAE)-salt eluate went to G 100 column. G-100 active pools from a similar separative sequence were separated by Concanavalin A chromatography and eluted with α -methyl mannose. Fractions from this separation had synergizing activity but no detectable protein by Bradford assay.¹⁵

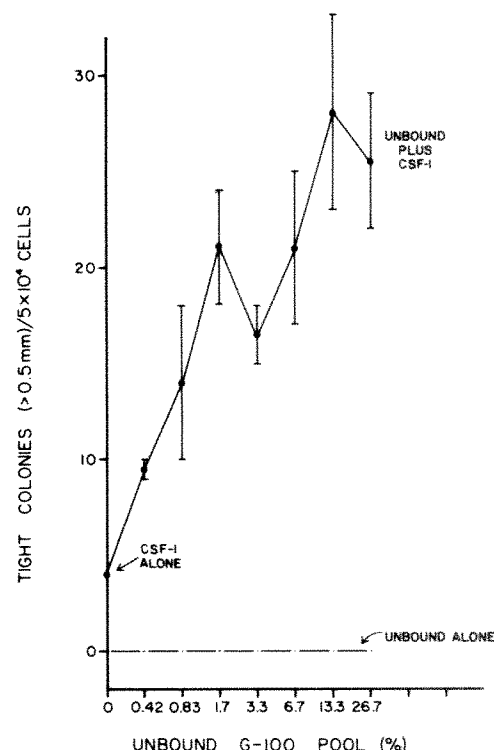


Fig 10. Synergistic activity of TC-1 conditioned media (CM) purified by diethylaminoethanol (DEAE) cellulose, Sephadex G-100 chromatography, and CSF-1 antibody affinity chromatography. TC-1 CM was separated by batch DEAE cellulose elution and G-100 Sephadex chromatography. Active fractions from the G-100 column were pooled and passed over an anti-CSF-1 affinity column. This "unbound" material was tested at various concentrations for its capacity to synergize colony formation by stage 1 CSF-1 derived from L cell CM. This latter material represents the eluate from a batch Ca phosphate gel¹⁷ and was included in single-layer agar cultures at 533 U/mL. Marrow cells from BDF-1 mice were plated at 5×10^4 cells/mL. Results are expressed as colonies per 5×10^4 cells $\pm$ 1 SEM from one of two separate lot testings that gave similar results.

Witte B lymphocyte cultures. Further work should clarify these alternatives.

We began a biochemical characterization of the synergizing activity using CSF-1 synergy as an assay. We found that the CSF-1 synergistic activity is nondialyzable and relatively heat stable, precipitates between 35% and 65% ammonium sulfate, and binds to DEAE cellulose under conditions in which IL 3 does not.¹⁴ Furthermore, the synergistic activity concentrates on Amicon PM100 membranes and is found near the void volume with both Sephacryl and Sephadex chromatography indicating a relatively high mol wt or, alternatively, the possibility of aggregate formation. This activity on chromatographic separations shows some polydispersity, and the bulk of the material appears to bind to Con A columns, eluting off with α -methylmannoside, indicating the presence of a glycoprotein although some of the material does not bind. Sequential separations through DE-52 cellulose chromatography and G100 Sephadex chromatography result in a 135-fold purification, and separation of this material or similar material on Con A columns results in

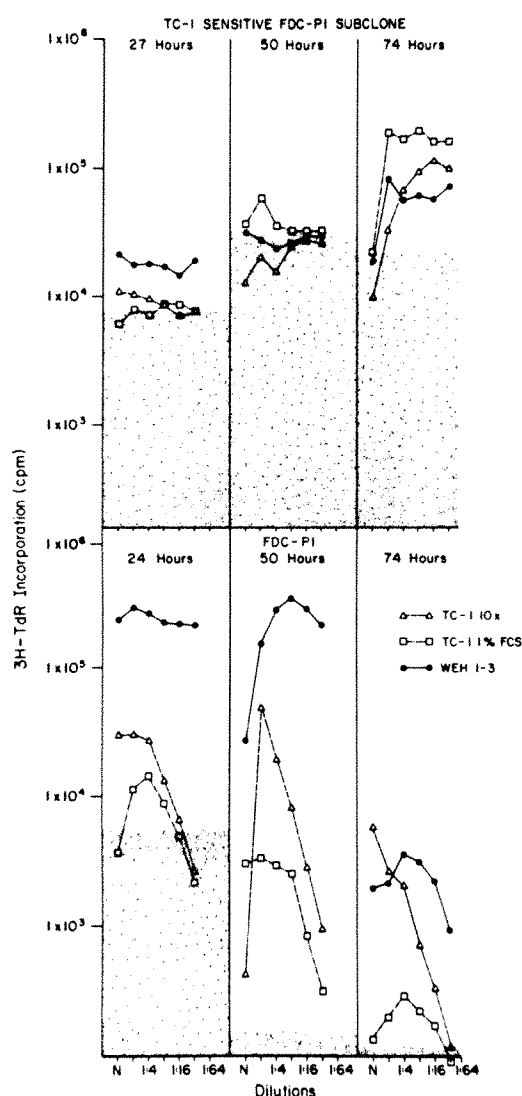


Fig 11. Stimulation of FDC-P1-sensitive and TC-1-sensitive FDC-P1 cells by conditioned media (CM) from the TC-1 cell line. FDC-P1 cells or FDC-P1 cells that had been cloned in soft agar in the presence of TC-1 CM and the clones then subcultured in liquid culture with 25% TC-1 CM (TC-1-sensitive FDC-P1 subclone) were tested for their responsiveness to WEHI-3 CM or to CM from TC-1 cells cultured with 1% fetal calf serum (FCS) and Nutridoma-SP supplement (TC-1 1% FCS) or to a 10 $\times$ concentrate of TC-1 CM originally cultured with 5% FCS. Cells were cultured at 6×10^5 cells/mL (TC-1-sensitive FDC-P1 subclone) or 1×10^6 cells/mL (FDC-P1) in RPMI media with 10% FCS for 27, 50, or 74 hours. Plates were pulsed with ^3H TdR (specific activity 6.7 Ci/mmol) 5 hours prior to harvest, and results expressed as cpm.

fractions with no detectable protein but with significant activity. Thus, the synergistic bioactivity appears to be a relatively large mol wt glycoprotein but, given the history of other hemopoietic glycoprotein hormones, the glycoprotein may well have a much smaller mol wt with further purification. Thus far in preliminary studies, the pre-B activity has been found in active fractions through the Con A separation step. The BCGF-2-like activity has also been found in active fractions but appears to partially separate with various chromatographic steps.

Finally, we also found an activity in the TC-1 CM and in partially purified fractions that stimulates the FDC-P1 and DA-1 factor-dependent cell lines.^{25,26} These data probably extend further the number of activities that can stimulate these factor-dependent lines. Hapel and colleagues²⁶ showed that these lines respond to multiple different activities, but they may provide a worthwhile tool for studies of the nature of the growth action and for a possible assay for its purification.

The TC-1-derived activity is similar to activities described previously^{27,28} in that it synergizes with CSF-1. These activities probably are represented by IL 3 and hemopoietin-1.^{3-5,29} The multilineage synergistic activity described from the TC-1 cell line does not appear to be IL 3, based on its lack of intrinsic colony-forming ability, a failure to be inactivated by an antibody to IL 3, and its behavior on DE-52 cellulose columns. It differs from hemopoietin-1^{4,29} in its apparent mol wt, lack of stimulation of total colony numbers in the presence of IL 3, and binding to Con A columns. The X factor described by Iscove³⁰ using a different system of synergy may well be similar to this activity. Recently, two separate activities predominantly stimulating B cell types were described, and both were termed IL 4.^{31,32} Different assays were used to define these factors but a comparison of both IL 4s to our activity will clearly be appropriate.

Finally, based on the above findings and considerations, we speculate that the long-term myeloid and lymphoid *in vitro* cultures are controlled by a series of growth factors. One unifying multilineage synergizing activity may exist for both B lymphopoiesis and myelopoiesis that determines responsiveness of cells to terminal differentiating hormones. We propose that the pre-B-inducing activity and the myeloid synergizing activities represent this activity. Furthermore, we suggest that in the myeloid Dexter cultures the production of CSF-1 by a particular stromal cell type with a secondary production of GM-CSA by stimulated macrophages determines the final lineages seen in this system; macrophages, granulocytes, and megakaryocytes. Similarly, in the Whitlock and Witte B lymphocyte culture system, we suggest that the production of factors acting on the terminal end of the B cell differentiation pathway modulates the production of B cells. The particular culture conditions used may well determine whether these terminal differentiating hormones are produced, thus providing a possible explanation of the switching from B lymphopoiesis to myelopoiesis in these cultures when conditions are altered.

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Value of Monoclonal Anti-CD22 (p135) Antibodies for the Detection of Normal and Neoplastic B Lymphoid Cells

By D.Y. Mason, H. Stein, J. Gerdes, K.A.F. Pulford, E. Ralfkiaer, B. Falini, W.N. Erber, K. Micklem, and K.C. Gatter

Two monoclonal antibodies (To15 and 4KB128) specific for the B cell-associated CD22 antigen (135,000 mol wt) are described. On immunoenzymatic analysis of cryostat tissue sections, these antibodies strongly label both mantle zone and germinal center B lymphoid cells in secondary lymphoid follicles (and also scattered extrafollicular lymphoid cells) but are unreactive with other cell types (with the exception of weak reactivity with some epithelioid histiocytes). These reactions differ from those of monoclonal antibodies B1 and B2 (anti-CD20 and CD21) but are similar to those of the pan-B antibody B4 (anti-CD19). One of the anti-CD22 antibodies (To15) has been tested extensively by immunoenzymatic labeling on >350 neoplastic lymphoid and hematological samples. The CD22 antigen was found in tissue sections in most B cell-derived neoplasms, the major

exceptions being myeloma (all cases negative) and a small proportion of high-grade lymphoma (6% of cases negative). In cell smears, the antigen could be found on neoplastic cells in most B cell lymphoproliferative disorders, including common acute lymphoblastic leukemia (ALL) (90% positive) and B cell chronic lymphocytic leukemia (CLL) (89% positive). We conclude that anti-CD22 antibodies are of value for identification of human B cell lymphoproliferative disorders (especially when used in conjunction with anti-CD19 antibodies). Previous reports that the CD22 antigen is absent from many B cell neoplasms are probably due to its being expressed within the cytoplasm of immature B cells rather than on their surface.

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WIDESPREAD use has been made in recent years of monoclonal antibodies for the immunocytochemical identification of lymphoid cell populations in human tissue and blood samples. The first of these reagents to be characterized were directed against T cell antigens, and at the 1st International Workshop on Human Leucocyte Differentiation Antigens (held in Paris in 1982) eight different T cell-associated molecules were defined.¹ Following the convention established at that Workshop, these molecules are prefixed by the letters CD and listed from 1 to 8.

An equivalent number of B cell-associated markers was not defined at the first Workshop, the only categories emerging at that time being CD9 (a 24,000 mol wt molecule shared between B cells and renal tissue) and CD10, the Common-ALL antigen (CALLA). A number of new B cell-associated molecules were, however, defined in the second Workshop (held in Boston in 1984) and numbered CD19 through 24.² Several of these appear to be restricted to B cells, whereas others are shared between B cells and dendritic reticulum cells (the antigen-presenting cell found in B cell follicles).³

Although these B cell-associated molecules are clearly defined entities, little information is available on their relative value as markers in the immunocytochemical detection of human B cell neoplasms. In the present article, we describe our experience in using two monoclonal antibodies against the 135,000 mol wt CD22 antigen for the detection of neoplastic B cells in cryostat sections and cell smears. It is

evident from this study that anti-CD22 antibodies can be confidently used as pan-B reagents for identification of B cell neoplasms, since they react with most such tumors and are unreactive with T cell-derived neoplasms.

MATERIALS AND METHODS

Tissues

Human tissue biopsies were obtained fresh from the Histopathology Department of the University Hospital, Kiel, and from the John Radcliffe Hospital, Oxford, England. Routine tonsillectomy samples were obtained from the Ear, Nose, and Throat Departments of these hospitals. Histological diagnosis was based on examination of paraffin sections stained with hematoxylin and eosin and/or Giemsa, lymphoid neoplasms being classified according to the Kiel system. Immunohistological phenotyping following immunoenzymatic staining of cryostat sections (described later) was performed as described elsewhere.⁴⁻⁶

Cell Samples

Smears of peripheral blood and bone marrow samples from the Hematology Department of the John Radcliffe Hospital and of normal blood from healthy laboratory personnel were stored at -20°C until they were labeled immunoenzymatically. In addition, cytocentrifuge preparations of Triosil-Ficoll-separated mononuclear cells were stored in the same way. Tonsil cell suspensions were prepared and, in some experiments, enriched in follicular dendritic reticulum cells (FDRCs), as described previously.⁷

In some experiments, cytocentrifuge preparations were made from normal peripheral blood mononuclear cells that had been rosetted with neuraminidase-treated sheep RBCs.

Immunocytochemical Reagents

Details of the monoclonal antibodies used in this study and of the immunoenzymatic reagents (eg, PAP, APAAP, peroxidase conjugates) are given in previous publications.^{5,6,8,9} Antibodies B1, B2 and B3 were obtained from Dr L. Nadler and from Coulter.

Immunoenzymatic Labeling Procedures

Tissue sections and cell smears were labeled by a two-stage or three-stage indirect immunoperoxidase procedure,¹⁰ by the PAP immunoperoxidase technique,⁸ or by the APAAP immunalkaline phosphatase technique.^{11,12}

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Production of Monoclonal Antibodies to the CD22 B Cell-Associated Antigen

Two antibodies of this specificity were prepared and used in this study.

Antibody To15. A cell fusion experiment was performed using spleen cells from a Balb-c mouse immunized with a potassium iodide extract of normal human tonsil cell membranes.^{13,14} Culture supernatants were screened by immunoperoxidase labeling of tonsil cryostat sections,¹⁵ and one clone (To15) reacting selectively with B cell follicles was cloned and grown by conventional procedures.¹⁴

Antibody 4KB128. Antibody 4KB128 was obtained (by the same hybridoma technique) using spleen cells from a mouse previously immunized with neoplastic cells from a case of hairy cell leukemia.¹⁶

Immunoprecipitation

Immunoprecipitation experiments were performed by conventional procedures, using surface-iodinated human tonsil cells and rabbit anti-mouse Ig bound to Staphylococcal protein A as precipitating reagent. Electrophoresis in sodium dodecyl sulfate (SDS) polyacrylamide gel and autoradiography were performed by conventional techniques.

Purification of Antigen

Membranes extracted from frozen hairy cell leukemia spleen in 2.5% Tween 40 were solubilized in 2% Nonidet P40, 20 mmol/L of Tris-HCl pH 7.5 containing protease inhibitors.¹⁷ The detergent extract was precleared by passage over bovine serum albumin (BSA) coupled to Sepharose 4B, and 25 mL (equivalent to 20 g of original tissue) was then passed down a 5 mL Sepharose 4B:4KB128 antibody column (antibody 4 mg/mL of Sepharose) at 1 mL/h. After being washed with 50 mL of 250 mmol/L of NaCl in 1% Nonidet P40, 20 mmol/L of Tris-HCl pH 7.5 at 2 mL/h, bound antigen was eluted with a 40-mL pH 7.5 linear gradient at 2 mL/h. Fractions were precipitated with 10% trichloroacetic acid (TCA), washed with acetone, solubilized in SDS-urea, and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) using a 7.5% gel and Coomassie blue staining.

RESULTS

Production of Monoclonal Antibodies To15 and 4KB128

Supernatant from two hybridoma cell cultures, To15 and 4KB128, reacted selectively with B cell follicles in lymphoid tissue, and the same pattern was maintained during cloning and subsequent growth of these cell lines.

Mol Wt of Antigen Detected by Antibodies To15 and 4KB128

Antibodies To15 and 4KB128 both precipitated a polypeptide chain with a mol wt under reducing conditions of 125,000 to 135,000. In some experiments, this material appeared as a doublet of two bands of only slightly different mol wts. It was also possible to isolate the CD22 antigen from human tissue using Sepharose antibody immunoabsorbents (Fig 1). This material migrated with the same mol wt as that of the radiolabeled band detected by immunoprecipitation.

Immunocytochemical Labeling of Peripheral Blood Cells

Immunoenzymatic staining of cell smears. Antibody To15 labeled 15% of lymphoid cells (range 10% to 25%) in

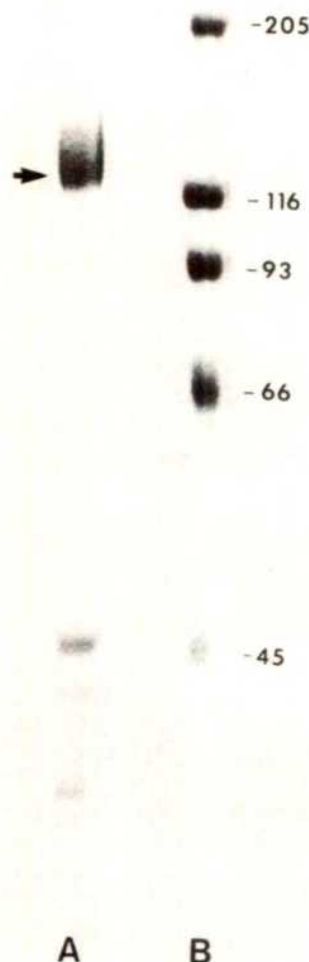


Fig 1. Demonstration of the specificity of antibody 4KB128 for the CD22 antigen by elution from an immunoabsorbent column (described in text). In track A, the prominent protein band (arrow) of ~125,000 mol wt represents the CD22 antigen. Small amounts of contaminating protein of lower mol wt are near the bottom of the gel. Track B shows the mol wt (in kD) of marker proteins run on the same gel. The mol wt of the CD22 antigen in this experiment was lower than the conventionally quoted figure (135,000) but in keeping with results reported by other authors²⁴ (Coomassie blue staining).

blood smears from 15 normal subjects. Antibody 4KB128 (tested on a smaller number of samples) labeled essentially the same number of lymphoid cells. Granulocytes, monocytes, RBCs, and platelets were unstained. In cytocentrifuge preparations of peripheral mononuclear cells rosetted with sheep erythrocytes, antibodies To15 and 4KB128 reacted selectively with nonrosetting lymphocytes.

Immunohistological Labeling of Human Tissue Sections

Normal tissue. The two anti-CD22 antibodies labeled B cell follicles with moderate to strong intensity (depending on the labeling procedure used) staining both mantle zone lymphocytes and germinal center cells. In centrifuged preparations of tonsil-cell suspensions enriched for FDRCs, antibody To15 labeled these cells weakly, although this could not

be discerned (because of lymphoid cell reactivity) in tissue sections.

The two anti-CD22 antibodies were compared with the pan-B reagents B1, B2, and B4 (anti-CD20, 21, and 19, respectively). Neither B1 nor B2 labeled B cell follicles as extensively as, or gave reactions of comparable intensity to, the two anti-CD22 reagents. Both B1 and B2 stained follicle mantle zone lymphocytes more weakly than germinal centers, and much of the germinal center staining was in a meshwork pattern in germinal centers characteristic of FDCs (particularly marked with antibody B2). In contrast, antibody B4 was closely similar in its staining reactions to the anti-CD22 reagents, labeling both mantle zones and germinal centers.

In addition to B cell follicles, antibodies To15 and 4KB128 stained scattered small cells in interfollicular and paracortical regions. The two antibodies were unreactive with all nonlymphoid tissue and organs tested (skin, lung, stomach, small and large intestine, kidney, liver, salivary and thyroid gland, urinary bladder, testis, and central and peripheral nervous tissue), with the exception of weak staining of epithelioid histiocytes in granulomatous lesions. Antibody B1 also reacted with epithelioid histiocytes, usually more intensely than the anti-CD22 reagents.

Neoplastic tissues. One of the two anti-CD22 antibodies (To15) was tested against a wide range of neoplasms (Table 1). It labeled most human B cell neoplasms, the principal exceptions being myeloma (consistently negative) and a few high-grade B cell lymphomas (4 negatives in 64 cases). The strength of labeling was usually of moderate intensity and could be enhanced markedly by use of the three-stage immunoperoxidase technique¹⁰ or the "enhanced" APAAP procedure.⁹ The strongest reactions were seen in hairy cell leukemia and B cell prolymphocytic leukemia. The intensity of labeling of cases of common acute lymphoblastic leukemia (common ALL) and B cell chronic lymphocytic leukemia (CLL) varied widely; a number of positive cases showed only weak labeling. Nevertheless, most cases in each of these two disorders gave unequivocally positive reactions.

Other neoplasms analyzed with antibody To15 (Table 1), were unreactive (including 56 cases of T cell lymphoproliferative disease) with the exception of a minority of cases of acute myeloid leukemia. These reactions were usually weak; in one case of acute monocytic leukemia, however, the neoplastic cells were relatively strongly labeled. The other anti-CD22 antibody (4KB128) was tested on a smaller number of neoplasms and gave reactions closely similar to those of antibody To15.

DISCUSSION

Both of the antibodies used in this study appear to recognize the B cell-associated molecule designated CD22. This is evidenced both by the results of immunoprecipitation and affinity chromatography experiments from this laboratory and by similar experiments and statistical analysis of FACS data in the 3rd International Workshop on Human Leucocyte Differentiation Antigens.¹⁸ The observation that in some experiments two polypeptide chains of similar mol wt

Table 1. Immunocytochemical Staining of Human Neoplasms With Antibody To15 (Anti-CD22)

Neoplasm	No. Tested	No. Positive
B cell derived		
Chronic lymphocytic leukemia	53	49
Prolymphocytic leukemia	7	7
Hairy cell leukemia	28	28
Lymphoplasmacytoid lymphoma/Waldenström's	9	8
Multiple myeloma	26	0
Centroblastic-centrocytic lymphoma*	22	22
Centrocytic lymphoma	18	18
Centroblastic lymphoma†	23	21
Lymphoblastic lymphoma, Burkitt-type	9	9
Lymphoblastic lymphoma, non-T, CALLA-positive	14	14
Immunoblastic lymphoma†	18	16
Common acute lymphoblastic leukemia	41	37
Acute lymphoblastic leukemia	1	1
Total	269	230
Other leukemias		
Acute myelomonocytic leukemia	15	2
Acute monocytic leukemia	4	1
Acute megakaryoblastic leukemia	6	0
Total	25	3
T cell derived		
Chronic lymphocytic leukemia (T cell)	11	0
Sézary syndrome/mycosis fungoides	6	0
Peripheral T cell lymphoma	25	0
Lymphoblastic lymphoma, thymic type	20	0
Lymphoblastic leukemia	12	0
Total	74	0
Miscellaneous		
Carcinoma	20	0
Fibrosarcoma	2	0
Osteosarcoma	1	0
Total	23	0

Staining was performed on cryostat tissue sections as described in the text, or by the APAAP immunoenzymatic procedure on blood or bone marrow smears, or on cytocentrifuge preparations. In all cases scored as positive, the majority (>70%) of neoplastic cells gave a positive reaction.

*Centroblastic-centrocytic lymphoma is approximately equivalent to follicular lymphoma in other classifications.

†Centroblastic and immunoblastic lymphoma are equivalent to "histiocytic lymphoma, diffuse" in the Rappaport classification.

were immunoprecipitated is in keeping with a similar report from Moldenhauer and colleagues on two other anti-CD22 antibodies.¹⁹

The two anti-CD22 antibodies clearly delineated B cell follicles in cryostat sections of lymphoid tissue and did so with greater clarity than did either B1 (anti-CD20) or B2 (anti-CD21). Hofman and co-workers²⁰ also found that antibody B1 labeled germinal center cells but only a proportion of mantle zone lymphocytes; in contrast, Bhan and co-workers²¹ reported labeling of all cells in lymphoid folli-

cles (both mantle zones and germinal centers). This discrepancy may relate to the relative sensitivities of labeling methods. When adjacent lymphoid tissue sections were stained with antibody B1 and anti-CD22 antibodies, however, the former reagent consistently gave weaker reactions. In this context, the initial detection of the two anti-CD22 antibodies by screening on tissue sections (rather than by binding to cell suspensions, used in screening for antibody B1) may account for their better reactivity as immunohistological reagents.

Anti-CD22 antibodies thus appear to be valuable reagents for labeling B cells in tissue sections and also in cell smears, and are comparable in our experience to antibodies against the 95,000 mol wt B cell-associated marker CD19 (first detected by antibody B4).²² They appear to be specific for B cells, with the exceptions of weak reactions with FDRCs and epithelioid histiocytes. Furthermore, the reaction of anti-CD22 with epithelioid histiocytes and FDRCs was less pronounced than the labeling of these cells by antibodies B1 and B2, respectively.

As shown in Table 1, positive labeling with the anti-CD22 antibodies was obtained in most B cell neoplasms in tissue sections or cell smears. The negative reactions of cases of myeloma is in keeping with observations that a number of other human B cell antigens are lost during terminal differentiation to plasma cells.² The only positive reactions against neoplastic cells other than B cells were those occasionally observed with cells from myeloid leukemia (Table 1). This may be related to the observation that CD22 is weakly

expressed by epithelioid histiocytes (ie, by another cell type of myeloid/monocytic origin).

The broad spectrum of reactivity of anti-CD22 antibodies against neoplastic B cells observed in the present study contrasts with other reports that the CD22 antigen is undetectable on many B cell neoplasms.² This discrepancy may be related to reports that the CD22 antigen is initially found during B cell maturation in the cell cytoplasm, and only subsequently emerges onto the cell surface.^{19,23} In consequence, blast cells in cases of common ALL contain cytoplasmic CD22 but lack this antigen on their surface.^{19,23} Phorbol ester stimulation of common ALL and pre-B cell lines can induce membrane expression of CD22, however.²³ The practical implication of these observations is that immunocytochemical staining methods that allow antibodies to gain access to the cell interior (ie, in fixed cell smears or tissue sections) will reveal cytoplasmic CD22, whereas staining of cells in suspension will only reveal surface membrane antigen.

Whatever the explanation of the higher positivity rate for CD22 antigen on neoplastic B cells in the present study as compared with previous reports,² it is evident that these monoclonal anti-CD22 antibodies will detect most B cell neoplasms studied in tissue sections or cell smears. Because identification of neoplastic cell lineage on the basis of at least two independent markers is advisable, we believe that in routine diagnostic practice a combination of anti-CD19 and CD22 antibodies constitutes a reliable pair of reagents for detecting B cell neoplasms.

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Evaluation of Circulating Malignant Cells Provides Prognostic Information in Cutaneous T Cell Lymphoma

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Peripheral blood lymphocyte morphology was evaluated prospectively by light microscopy of blood smears and E rosette preparations in 160 patients with cutaneous T cell lymphoma (CTCL). Blood involvement was related to the type of cutaneous T-stage, being present in 90% of patients with erythroderma (T4), 27% of those with cutaneous tumors (T3), 9% of those with generalized (T2), and 0% of those with limited skin plaques (T1). Untreated patients with blood involvement (38 of 105) had a higher frequency of CTCL in lymph nodes and viscera and survival inferior to that of patients with normal or nondiagnostic lymphocyte morphology ($P < .001$). Multivariate analysis showed skin stage and age to be the most important pretreatment risk factors for survival. Although blood involvement was not an independent risk factor for the

entire group, it appeared to have some adverse influence in the T2/T3 subsets ($P = .051$). Both lymphocytosis and size distribution of the circulating CTCL cells at initial diagnosis influenced survival. Patients with "mixed cell" cytology ($>20\%$ large [$>11 \mu\text{m}$] CTCL cells), had a worse survival than those with predominantly small circulating CTCL cells ($P = .009$). The former were more likely to have aggressive features, including lymph node effacement by tumor ($P < .001$) and visceral disease ($P = .074$), than were "small cell" patients. Our data indicate that detailed review of the blood lymphocyte morphology in patients with diagnosed or suspected CTCL is helpful in predicting extent of disease and prognosis.

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FOR MANY YEARS, mycosis fungoides (MF) was considered primarily a cutaneous disorder, with plaques, cutaneous tumors, and erythroderma recognized as types of skin involvement.¹ In 1938, Sézary and Bouvraïn described a group of patients with generalized erythroderma who had large, bizarre mononuclear cells in the peripheral blood.² Similarities between the erythrodermic form of MF and the Sézary syndrome (SS) were soon recognized. In 1970, Crossen and colleagues showed that the abnormal cells of SS were malignant lymphocytes³; subsequently, both MF and SS were shown to be malignant proliferations of mature helper T cells.⁴⁻⁷ Because of their similar clinical and histopathologic features, the term cutaneous T cell lymphoma (CTCL) has been used to include both MF and SS^{8,9} in a spectrum of disease. At one end of the spectrum is non-erythrodermic MF limited to the skin; at the other extreme is SS with erythroderma and frank leukemia.

Using light microscopy, Clendenning and colleagues reported that up to 20% of advanced plaque/tumor MF patients without erythroderma had circulating convoluted cells similar to those of SS.¹⁰ Other investigators reported that these cells could be identified by cytologic features and electron microscopy as well as by cytogenetic and DNA content analyses.¹¹⁻¹⁵

Several studies addressed the clinical implications of blood involvement¹⁶⁻¹⁹; however, systematic evaluation prior to therapy was not fully explored until recently. Vonderheid and colleagues showed that prognosis was influenced adversely when there were $>10\%$ Sézary cells¹⁷ or increased proportions of large convoluted cells.¹⁹ We previously reported that peripheral blood involvement had significant impact on prognosis in a small group of prospectively staged patients.^{20,21} That series has now been extended to 160 patients with CTCL to define the frequency, extent, and morphological variety of circulating CTCL cells detected by light microscopy and to determine the importance of these features for prognosis.

METHODS AND MATERIALS

Patients. The clinical features of the 160 CTCL patients studied by the NCI-Navy Medical Oncology Branch and the Hematology,

Dermatology, or Medical Oncology Sections of the Washington Veterans Administration Medical Center between June 1975 and March 1984 are shown in Table 1. Informed consent for the staging procedures and subsequent treatment was obtained for all patients according to the policies of the human studies review boards of the National Naval Medical Center or the Veterans Administration Medical Center and the National Institutes of Health. Group I comprised 105 patients who were staged within 12 months (median 1.4 months) of initial diagnosis and had received no systemic chemotherapy prior to staging. The remaining 55 patients (group II) had received prior chemotherapy or were staged >1 year from diagnosis and were not evaluated for survival. Group I patients were entered into two sequential CTCL treatment protocols. The first 39 patients were treated with total-skin electron-beam radiotherapy and chemotherapy²²; the remaining patients were randomized to either intensive combined modality therapy or sequential palliative therapies.²³ Group I patients were followed for a median of 54 months.

Staging evaluation. One dermatologist (A.B.F.) characterized the skin lesions using the TNM classification for cutaneous T cell lymphoma.²⁴ In the first 40 patients, a lymph node was biopsied only if lymphadenopathy existed. Subsequently, blind biopsies were also attempted in patients without palpable nodes. The histopathologic

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Table 1. Clinical Features of CTCL Subjects

Characteristic	Group I*	Group II†	Total
No. of subjects	105	55	160
M/F	73/32	30/25	103/57
White/black	73/31	44/11	117/42
Age at diagnosis (yr), mean (range)	53 (22–75)	50 (19–75)	52 (19–75)
Time from diagnosis to staging (mo), median (range)	1.4 (<1–12)	34 (<1–176)	2.8 (<1–176)
Skin class n (%)			
T1, limited plaque	13 (12)	4 (7)	17 (11)
T2, generalized plaque	35 (33)	22 (40)	57 (36)
T3, skin tumors	22 (21)	13 (24)	35 (22)
T4, erythroderma	35 (33)	16 (29)	51 (32)

CTCL, cutaneous T cell lymphoma.

*Group I patients were staged within 12 months of diagnosis and had received no systemic cytotoxic chemotherapy prior to evaluation of lymphocyte morphology.

†Group II patients had received prior systemic chemotherapy and/or had been diagnosed > 12 months before evaluation or were lost to follow-up after initial studies.

findings of the lymph node biopsies were classified according to the number of atypical cells in paracortical node areas and the degree of node effacement.²⁵ This classification describes node involvement as LN1 (no atypical cells), LN2 (small clusters of atypical cells), LN3 (large clusters of atypical cells), and LN4 (partial or total effacement of normal architecture by malignant cells). The initial staging evaluation also included a chest x-ray, lymphangiogram, liver biopsy, and bone marrow biopsy. In the last 60 patients, liver biopsy was performed only if adenopathy, LN3 or LN4 node histopathology, or blood involvement existed.

Peripheral blood studies. Peripheral blood lymphocyte morphology was evaluated by one hematologist (G.P.S.) using light microscopy at $\times 1,500$ magnification without knowledge of the type of skin lesions or other physical findings. Every patient had a WBC count and a differential count of a Wright's-Giemsa-stained smear that included evaluation of the nuclear morphology of at least 50 lymphocytes in areas devoid of rouleaux formation. In 145 patients, blood mononuclear cells were isolated by Ficoll-Hypaque centrifugation and the percentage of cells binding AET-treated sheep red cells (SRBCs) (133 patients) or unmodified SRBC (12 patients) was determined.^{26,27} The morphology of 200 E rosetted T lymphocytes was then examined on Wright's-Giemsa-stained cytocentrifuge preparations. Fluorescent antiimmunoglobulin antisera (Meloy, Springfield, VA) and fluorescence microscopy were used to determine the percentage of B cells in 90 patients.

The criteria for the diagnosis of CTCL infiltration of the blood were developed during our initial comparisons of lymphocyte morphology of CTCL patients with that of normal individuals and patients with nonmalignant skin disorders or lymphopenia.²⁰ Atypical cells were grouped in the following categories, except for lymphoid cells with cytoplasmic granules which were excluded. Category 1 describes small convoluted lymphocytes, cells $\leq 11 \mu\text{m}$ in diameter, with a nuclear density similar to or greater than that of normal lymphocytes and having nuclear grooves, cleaves, convolutions, or folds (Fig 1a through c). Category 2 describes large convoluted lymphocytes, cells $> 11 \mu\text{m}$ in diameter with nuclear abnormalities as described in category 1 or with dense hyperchromatic nuclear chromatin (Fig 2a,b). Category 3 describes "atypical" nonconvoluted lymphocytes, cells $> 11 \mu\text{m}$ without nuclear irregularities or convolutions but containing fine nuclear chromatin and/or nucleoli (blastlike or "transformed" cells, Fig 2d, arrow).

A diagnosis of blood involvement by CTCL was made if the sum of the convoluted cells (categories 1 and 2) was $\geq 20\%$ of the

lymphocytes in a blood smear and/or $\geq 10\%$ of the lymphocytes in the E-rosetted cytocentrifuge preparations (ERCs). Category 3 cells were generally not considered diagnostic, except in one patient with marginal numbers of convoluted cells and numerous nonconvoluted tumor cells.

Thirteen blood smears and 15 ERC preparations from 21 patients with $< 4,000$ lymphocytes/ μL , had cells that resembled the small convoluted cell type (category 1) but convolutions were minimal (single folds or small clefts), and category 2 cells were rare or absent. These patients were considered to have abnormal but nondiagnostic lymphocyte morphology. Thus, three groups of patients were identified with respect to their lymphocyte morphology: (a) positive for CTCL, (b) abnormal but nondiagnostic for CTCL, and (c) within normal limits. For purposes of analysis, patients with positive peripheral lymphocyte morphology were in all cases compared with the combined negative and abnormal but nondiagnostic patients.

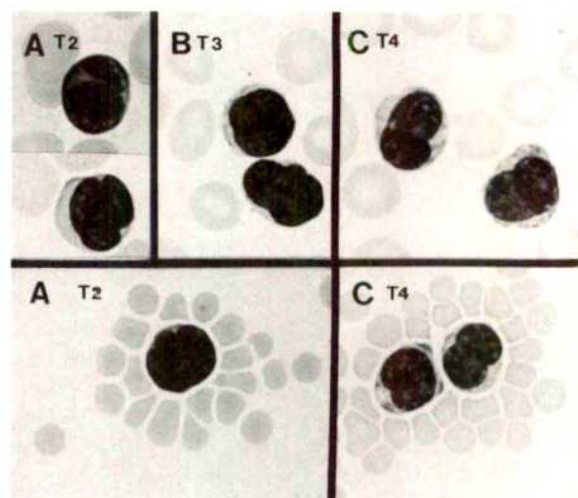


Fig 1. Peripheral blood lymphocytes in smears and E rosette preparations from three patients with cutaneous T cell lymphoma (CTCL) of the small cell type, original magnification $\times 1,400$; current magnification $\times 910$: (A) mycosis fungoides (T2); (B,C) erythrodermic CTCL (T4). The morphology of the CTCL cells tended to be uniform, with the diameter of $> 90\%$ of the cells between 8.0 and $10.5 \mu\text{m}$.

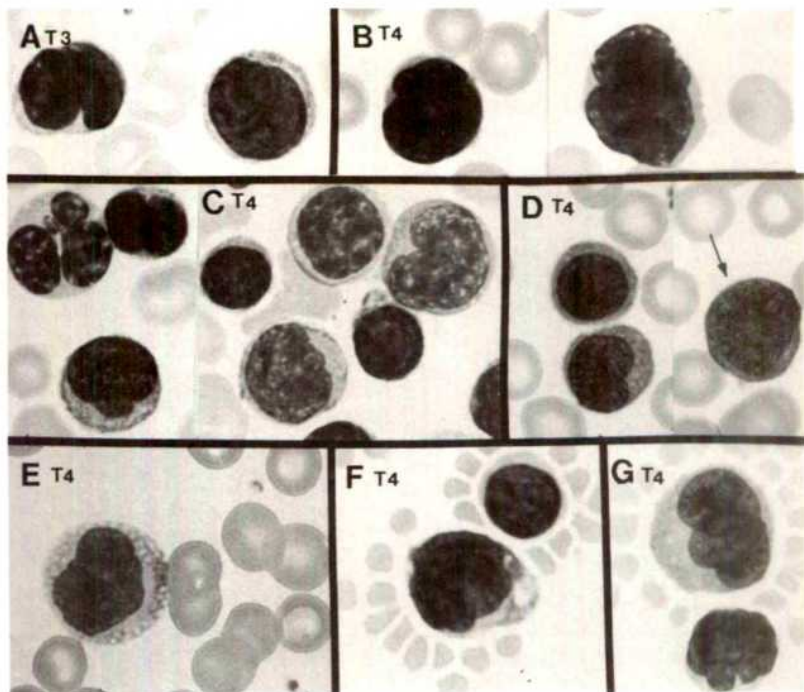


Fig 2. Peripheral blood lymphocytes on smear and E rosette preparations from five patients with cutaneous T cell lymphoma (CTCL) of the mixed cell type, original magnification $\times 1,400$; current magnification $\times 910$: (A) mycosis fungoides (T3); (B–E) erythrodermic CTCL (T4). The CTCL cells were highly pleomorphic and varied between patients and within the same patient. The diameters ranged between 8 and 17 μm in diameter. Tumor cells containing densely hyperchromatic nuclei (A,B), hyperconvoluted nuclei (B,E,F), nuclei with finely dispersed chromatin without convolutions (D, arrow) or only with vague grooving and rarely with nuclear segmentation were noted.

In patients with positive blood, two groups were identified on the basis of the size distribution of the convoluted cells, a mixed-cell variant and a small-cell variant. Mixed-cell patients had a ratio of category 1 to category 2 cells of <4 , with the percentage of large convoluted cells varying between 22% and 67% of all convoluted cells. In the small-cell patients, the ratio was >4 , and the median percentage of small convoluted cells was 94%. Tumor cells in the small-cell patients were fairly uniform, with round nuclei and subtle to distinct nuclear grooves, simple folding, or figure 8 shapes (Fig 1). Cells $>12 \mu\text{m}$ were rare. Mixed-cell patients demonstrated greater variability in cytologic characteristics. Frequently, a wide range of cell diameters from 8 to 17 μm existed in the individual patient, with a few patients having predominantly large ($>11 \mu\text{m}$) cells. Nuclei were often ovoid, with highly bizarre and pleomorphic convolutions and dense chromatin (Fig 2). In some mixed-cell patients, the tumor cells had more finely divided nuclear chromatin and prominent nucleoli (Fig 2d). Cytoplasm was usually scant in the small-cell patients and frequently abundant in the mixed-cell patients. Occasionally, extensive vacuolation occurred in both variants.

The light microscopic findings were compared with those made independently by J.G. using electron microscopy in 78 consecutive patients. The concordance between the light-microscopic and electron-microscopic interpretations was 84%. In 2 of 33 instances, a positive light-microscopic interpretation was not confirmed by electron microscopy; one of these cases was an erythrodermic patient with a frank leukemia of mature T cells with minor nuclear folding. Eleven patients positive by electron-microscopic criteria ($>6\%$ convoluted cells) were negative (5 patients) or abnormal/nondiagnostic (6 patients) by light microscopy. For the clinical analyses here, these 11 patients were retained in the negative or nondiagnostic groups. The electron-microscopic evaluation of the first 49 patients has been published.²⁸

Control subjects. During this study, the lymphocyte morphology of 71 individuals with diagnoses other than CTCL were also evaluated. This group included 28 normal subjects, 25 subjects with acute and chronic skin disorders, 13 patients with B cell lymphopro-

liferative disorders, and 6 patients with lymphopenia. More than 50% of the preparations from the control subjects were evaluated without knowledge of the diagnosis, interspersed among groups of slides from CTCL patients.

Statistical methods. Comparative analysis of discrete data was performed using standard chi-square tests, corrected for continuity where appropriate. Comparisons between groups of continuous data were made using the nonparametric Kruskal-Wallis one-way analysis of variance.²⁹ Survival curves were estimated from the date of diagnosis to the most recent follow-up date/death date using the Kaplan-Meier method.³⁰ Patients dying from any cause were considered failures, ie, uncensored observations. The method described by Mantel³¹ was used to compare survival curves unadjusted for any covariates. Once these univariate analyses were completed, a multivariate analysis was undertaken to determine the relative importance of several factors, simultaneously using the life-table regression approach of Cox.³² A ranking of the relative importance of these factors was established using both a forward step-up approach and a backward step-down approach. All *P* values in this report correspond to two-sided significance tests. Both approaches gave the same final set of independent and important prognostic factors.

RESULTS

Concordance of the peripheral smear and ERC evaluation in CTCL. Sixty patients (38%) were identified as having one or both preparations (blood smear or ERC) diagnostic for CTCL blood involvement. The remaining patients had either normal lymphocyte morphology (79 patients, 48%) or abnormal/nondiagnostic morphology on either the smear or ERC (21 patients, 14%). Of 145 patients studied, 45 had both positive smear and ERC preparations. The percentage of convoluted cells on smear (Fig 3) correlated significantly with the percentage of convoluted T cells noted on the ERC preparations ($P < .001$). In five patients, one of the preparations was considered positive (ERC 4,

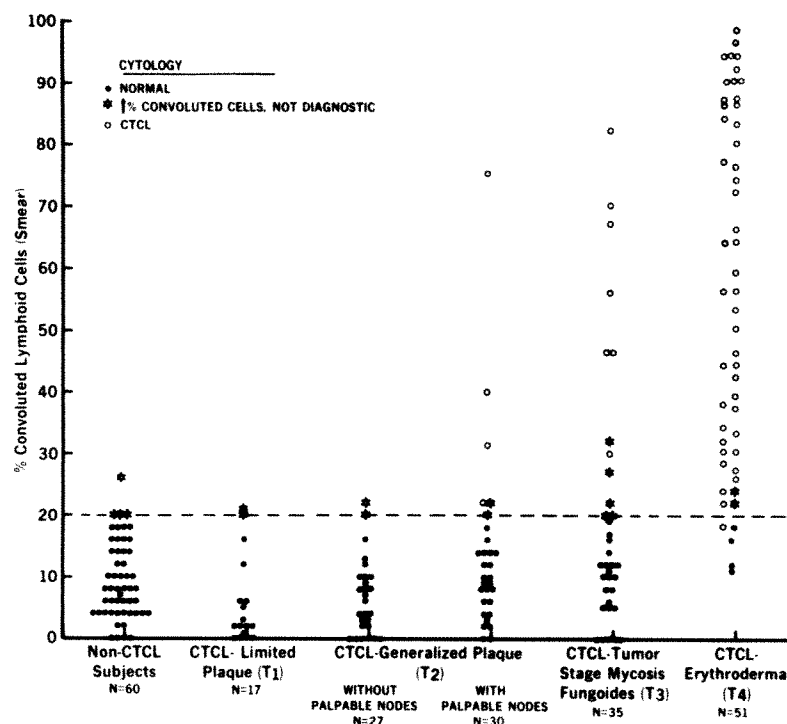


Fig 3. Percentage of convoluted lymphoid cells per 100 lymphocytes in peripheral blood smears in 60 subjects with noncutaneous T cell lymphoma (non-CTCL) and 160 CTCL subjects grouped according to skin stages T1 through T4 and palpable adenopathy in T2. The percentage of convoluted E rosetted cells in the 145 CTCL patients who also had E rosette preparations (data not shown) correlated significantly with the percentage of convoluted cells on smear ($P < .001$).

smear 1) whereas the other was not. Two patients with positive smears had inadequate ERC preparations due to extremely low percentages of T cells ($\leq 12\%$), and eight did not have an ERC study.

An abnormally low percentage of T cells ($< 58\%$ AET- or $< 45\%$ unmodified E rosettes) occurred in 11% of the patients and was associated with blood involvement in 13 of 17 instances. Eleven patients had a low percentage of B cells ($< 4\%$); B cell lymphopenia was apparently due to dilution in six patients with frank CTCL cell leukemia. It was unexplained in five patients with normal lymphocyte counts, three of whom had peripheral blood involvement.

No preparation from the 71 non-CTCL subjects, including 60 blood smears and 24 ERC preparations, met the criteria for infiltration by CTCL cells. Sixty-six non-CTCL subjects had completely normal lymphocyte morphology. Five non-CTCL subjects (7%) had abnormal lymphocyte morphology (smear 4, ERC 2) associated with diagnoses of follicular lymphoma, reaction to phenytoin, severe lymphopenia, and lymphomatoid granulomatosis (Fig 3).

Correlation of circulating CTCL cells with skin, node, and visceral disease. Diagnostic blood involvement was significantly associated with advanced skin disease (Table 2 and Fig 3), with a similar trend for patients with abnormal but not diagnostic morphology. Overall, 90% of erythrodermic (T4) and 13% of the nonerythrodermic (T1 through T3) patients had positive smears and/or ERC preparations. The erythrodermic patients with normal (three patients) or abnormal but not diagnostic (two patients) morphology were all moderately to severely lymphopenic (540 to $1,200/\mu\text{L}$). In patients without erythroderma, blood involvement was always associated with advanced disease, as judged by the presence of adenopathy (if T2) or cutaneous tumors (T3).

No patient with limited plaque disease or generalized plaque without palpable adenopathy had circulating CTCL cells detected by light microscopy. There was no difference in the frequency of blood involvement between group I (no prior treatment) and group II (prior treatment or delayed staging).

The association of blood involvement at initial diagnosis of CTCL (group I patients only) with lymphadenopathy, lymph node histology, and visceral involvement is shown in Table 3. Patients with blood involvement were more likely to have adenopathy ($P = .002$), lymph node involvement (LN3 or LN4) ($P < .001$), and visceral disease ($P < .001$) than were patients with normal or nondiagnostic lymphocyte morphology. Visceral involvement and lymph node effacement (LN4)

Table 2. Correlation of Skin Stage With Circulating CTCL Cells

Patients	No. With Circulating CTCL Cells (%)		
	Group I*	Group II*	Total
T1†	0/13 (0)	0/4 (0)	0/17 (0)
T2			
Adenopathy absent	0/17 (0)	0/10 (0)	0/27 (0)
Adenopathy present	3/18 (17)	2/12 (17)	5/30 (17)
T3	4/22 (18)	5/13 (38)	9/35 (27)
T1-T3	7/70 (10)	7/39 (18)	14/109 (13)
T4	31/35 (89)	15/16 (94)	46/51 (90)
Total (T1-T4)	38/105 (36)	22/55 (40)	60/160 (38)

CTCL, cutaneous T cell lymphoma.

*Group I, no prior treatment; group II, prior treatment or delayed staging.

†T refers to the TNM²⁴ staging system for cutaneous T cell lymphoma: T1, $< 10\%$ plaque; T2, $> 10\%$ plaque; T3, cutaneous tumors; T4, erythroderma.

Table 3. Correlation of Blood Involvement With Lymph Node and Visceral Disease at Initial Diagnosis of CTCL in Group I

Blood	No. of Patients (%) With						
	Palpable Adenopathy		Node Histology*			Visceral Involvement†	
	Present	Absent	LN1/2	LN3	LN4	Positive	Negative
Positive (n = 38)	32 (84)	6 (16)	3 (9)	15 (43)	17 (49)	14 (33)	22 (67)
Negative (n = 67)	36 (55)	30 (45)	31 (54)	25 (44)	1 (2)	5 (8)	60 (92)
P	.002		<.001			<.001	

CTCL, cutaneous T cell lymphoma.

*LN1/2 nodes were not diagnostically involved by CTCL. LN3 and LN4 nodes were infiltrated with CTCL, with LN4 nodes showing effacement of the normal architecture.²⁵

†Liver or marrow biopsy positive.

were uncommon in patients with negative blood findings (5 of 65 and 1 of 57, respectively).

Correlation of lymphocyte number and morphology with other prognostic features in patients with positive peripheral blood. The correlation of lymphocyte concentration, proportion of convoluted cells, and cell size distribution with skin class for all patients (groups I and II) with positive peripheral blood is shown in Table 4. Erythrodermic patients (T4) were significantly ($P = .043$) more likely to have high lymphocyte counts than were plaque/tumor (T2/T3) patients and a higher proportion (>20%) of large cells

Table 4. Lymphocyte Concentration and Cell Morphology Related to Skin Class in Patients With Circulating CTCL Cells

Factor	Skin Class		P
	T2,3 (n = 14)	T4 (n = 46)	
Lymphocyte concentration (per μL)			
<4,000	12 (86)*	22 (48)	
4,000–10,000	1 (7)	11 (24)	.043†
>10,000	1 (7)	13 (29)	
Cell size distribution			
Small cell type	11 (79)	22 (48)	.043†
Mixed cell type	3 (21)	24 (52)	
Convoluted cells (%)			
Median (range)			
Smear	39 (5–82)	62 (11–98)	.025‡
ERC	35 (18–70)	47 (9–99)	.11‡

CTCL, cutaneous T cell lymphoma; ERC, E-rosetted cytocentrifuge preparations.

Group I and group II are inclusive.

*Number of patients (%).

†Chi-square test.

‡Mann-Whitney test.

(mixed-cell type) ($P = .043$). T4 patients also had higher proportions of convoluted cells noted on smear than did the T2/T3 patients ($P = .025$).

The correspondence between lymphocyte count or cell size and lymph node biopsy findings and visceral disease in group I patients is shown in Table 5. Patients with small CTCL cells were unlikely to have node effacement at initial diagnosis of CTCL, whereas 83% of patients with mixed-cell type had lymph node effacement (LN4). Visceral involvement at diagnosis was more closely associated with high lymphocyte counts (>4,000/ μL) and, to a lesser extent, with the mixed-cell type ($P = .015$ and $P = .074$, respectively). Lymph node effacement (LN4) was also associated with higher lymphocyte counts but more particularly with the mixed-cell type ($P = .069$ and $P < .001$, respectively). In group I patients, neither type of skin disease nor adenopathy was associated with absolute lymphocyte counts or with cell size.

Mixed-cell patients had a higher frequency of lymphocytosis than did small-cell patients (12 of 20 v 4 of 18, $P = .061$). They also had higher percentages of convoluted lymphocytes and nonconvoluted tumor cells on smear ($P = .008$ and $P = .003$, respectively) and a lower fraction of lymphocytes with normal morphology (in patients with <6,000/ μL , $P = .046$) (Table 6).

Prognostic significance of peripheral blood findings. The actuarial survival of group I patients with diagnostic blood findings is shown in Fig 4. Patients with positive blood, either by smear or cytology, had a survival inferior to that of patients without positive blood ($P < .001$), with estimated 5-year survivals of 22% and 76%, respectively.

Lymphocyte size influenced survival in the patients with positive lymphocyte morphology. The actuarial survival of the mixed-cell patients was significantly shorter than that of patients with the small-cell type (23 v 51 months, $P = .009$;

Table 5. Correlation of Lymphocyte Count and Morphology With Lymph Node and Visceral Involvement in Positive Group I Patients

Factor	Visceral Involvement			Node Histology			P
	+	–	P	LN2	LN3	LN4	
Lymphocyte concentration (μL)							
>10,000	6	1		0	1	6	
4,000–10,000	3	5	.015	0	4	4	.069
<4,000	5	16		3	10	7	
Cell size							
Small (n = 18)	4	13	.074	3	12	2	<.001
Mixed (n = 20)	10	9		0	3	15	

Table 6. Concentration and Proportions of CTCL Cells and Normal Lymphocytes at Initial Diagnosis of Small-Cell and Mixed-Cell Patients

Factor	Small-Cell Patients (n = 18)	Mixed-Cell Patients (n = 20)	P
Lymphocyte concentration			
Median per microliter (range)	2,500 (1,300–93,000)	4,575 (870–120,000)	.041*
Proportion of convoluted cells†			
Median % (range)			
Smear	38 (5–98)	73 (30–96)	.008*
ERC	38 (13–76)	47 (18–99)	.27*
Proportion of nonconvoluted cells > 11 μ m†			
Median % (range)	5 (0–12)	11 (0–54)	.003*
Proportion of normal lymphocytes‡			
Median % (range)	38 (8–72)	16 (3–58)	.046*

Abbreviations as in Table 4.

*Mann-Whitney test.

†Per 100 lymphocytes.

‡In patients with <6,000 lymphocytes/ μ L.

Fig 5A). Similarly, Fig 5B shows that patients with small-cell CTCL involvement of blood and <4,000 lymphocytes/ μ L had a significantly improved survival in comparison with mixed-cell patients with <4,000/ μ L ($P = .025$) or all patients with abnormal cells >4,000/ μ L ($P = .032$).

Multivariate analysis of prognostic factors. To examine the effect on survival of several parameters simultaneously, the Cox life-table regression method was used in conjunction with two distinct ways of determining what variables would form the final statistical model. For the first approach, all variables given in Table 7 and examined in a univariate analysis were included. Using standard likelihood ratio procedures, a backward step-down approach was used to eliminate those variables found not to be significantly associated with survival at the .05 significance level.

Age, skin class, lymph node histology, adenopathy, and visceral disease as well as blood involvement were each significant predictors of inferior survival when analyzed in a

univariate fashion (Table 7). In the multivariate analysis, the only significant factors were skin class and age ($P < .001$ and $P = .025$, respectively). A forward step-up procedure was used as the second approach and resulted in the selection of these same two factors. With these two variables in the model, all remaining variables were not significant at the $P = .05$ level. Thus, T-class and age remained the most important and independent prognostic indicators of survival, with T1 and T2 patients and those patients aged <50 years associated with a favorable prognosis. Adjusted Mantel-Haenszel analyses also were used to confirm these findings.

Blood involvement was not a significant factor in the final model once age and T-class were included ($P = .31$). This finding is readily explained by the fact that blood involvement and T-class are highly associated ($P < .001$). The importance of age in this model remains although almost all deaths in this series were relatable to progressive cutaneous T cell lymphoma. Further analyses were made to determine if first including blood involvement in the model would eliminate the need for subsequently introducing T-class or age into the model. Because both T-class and age still entered the model, however, blood involvement appears not to have been an independent prognostic factor for the entire group of patients. In the subset of patients with T2 (generalized plaque) and T3 (cutaneous tumor) skin disease, however, blood involvement was ($P = .051$) associated with inferior prognosis (Fig 6). Although the number of patients in the nonerythrodermic, blood-positive subgroup was small, their inferior survival reinforces the potential usefulness of blood involvement in the prospective evaluation of this subgroup of patients with CTCL.

DISCUSSION

In this study of a large number of patients with cutaneous T cell lymphoma, we found that peripheral blood infiltration correlated with established prognostic factors, including advanced skin disease, palpable adenopathy, lymph node and visceral involvement, and older age. In this series, no patient with limited plaque disease or generalized plaques without

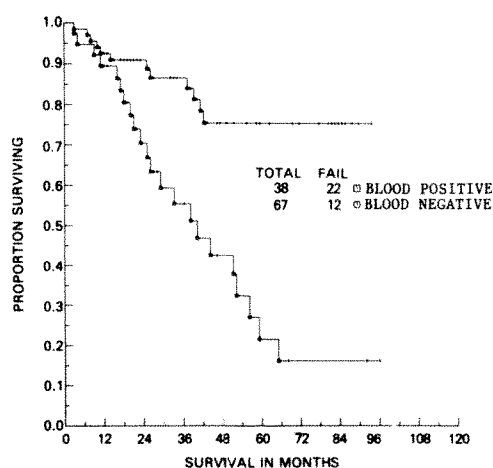


Fig 4. Actuarial survival of group I cutaneous T cell lymphoma (CTCL) patients with normal or nondiagnostic peripheral blood morphology (blood negative) differed significantly from that of patients with circulating CTCL cells (blood positive); ($P < .001$).

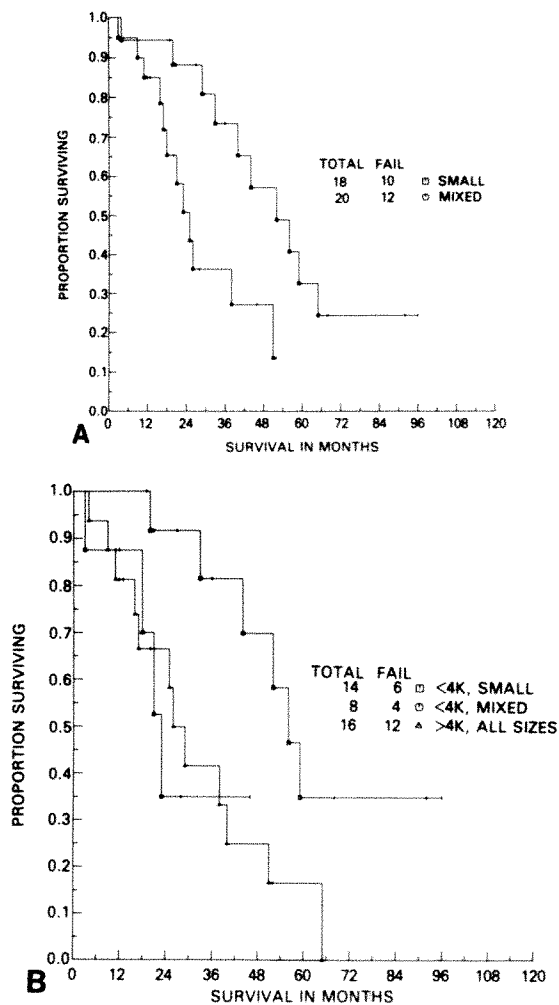


Fig 5. (A) Actuarial survival of group I cutaneous T cell lymphoma (CTCL) patients with blood involvement of the small cell type differed significantly from that of patients demonstrating >20% large cells (mixed cell type), ($P = .009$). (B) Actuarial survival of small cell type and mixed cell type patients according to lymphocyte concentration. Survival of patients with small cell CTCL with <4,000 lymphocytes/ μL differed from that of patients with mixed cell type with <4,000/ μL ($P = .015$) and from all patients with >4,000/ μL ($P = .032$).

adenopathy had blood involvement. Approximately 22% of plaque patients with adenopathy and/or skin tumors had blood involvement on detailed review of the peripheral blood smear or T cell cytology. In contrast, 90% of those with erythroderma had blood infiltration. In patients with blood involvement, we further identified >20% large convoluted cells or an absolute lymphocytosis as poor prognostic features.

The original description of SS directed attention to the importance of peripheral blood involvement in this disorder.² Many years later, using electron microscopy, Lutzner and co-workers recognized considerable variation in the size of Sézary cells and described a variant in which small cells predominated.¹¹ The recognition that mycosis fungoides and the Sézary syndrome were related disorders, malignancies of mature helper T cells, led to the discovery of circulating

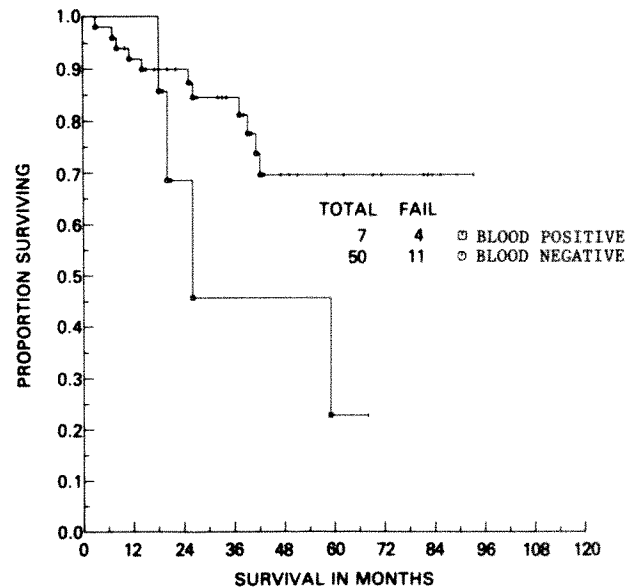


Fig 6. Actuarial survival of plaque and tumor (T2 and T3) cutaneous T cell lymphoma (CTCL) patients with blood involvement was inferior to those without blood involvement ($P = 0.051$).

“mycosis cells” in ~20% of mycosis fungoides patients.^{10,14,18,32,33,34} Correlation with other clinical and pathologic findings and prognosis have been addressed, however, in only a few of these studies.¹⁶⁻¹⁹ Our study affirms that the frequency of blood involvement is directly correlated with the type of skin lesion and other features of advanced stage. Tumor cell morphology did not differ between the erythrodermic (T4) or classic Sézary syndrome patients and the T2/T3 mycosis fungoides patients except that lymphocytosis and the mixed-cell type was more prevalent in the T4 patients. The presence of circulating tumor cells in a univariate analysis was associated with an inferior survival; however, multivariate analysis established that whereas both skin class and age >50 years were independent risk factors in this series, peripheral blood involvement was not. When T2 and T3 skin disease patients were evaluated separately, blood involvement appeared to affect survival adversely.

When Lutzner and co-workers described the small-cell variant Sézary cell, no prognostic significance was attributed to cell size. In the B cell lymphomas, nearly all studies show that large cell size is associated with a more aggressive natural history.³⁵ In this study, we demonstrated that CTCL patients with >20% of large convoluted cells (mixed-cell type) have shorter survival than those with blood involvement having predominantly small cells.

The mixed-cell type was strongly associated with features of aggressive disease, including lymph node effacement, visceral involvement, increased proportions of convoluted cells, and higher absolute concentrations. Vonderheid and colleagues recently reported that survival in a group of erythroderma patients was more closely related to the proportion of large convoluted cells (15 to 20 μm) than to the relative or absolute number of convoluted cells.¹⁹ Our data support earlier results of Vonderheid and colleagues¹⁷ in that

Table 7. Pretreatment Characteristics and Their Relation to Survival for Group I Patients (n = 105)

Characteristic and Description	No. of Patients in Analysis	Group (Observed/Expected)*	Univariate P Value
Age (<50 v ≥50)	101	Age <50 (7/15.54)	.003
Sex	105	Male (23/23.72)	.790
Race	104	White (24/22.99)	.710
Diagnosis to staging (<1 month v ≥1 month)	105	<1 month (18/17.14)	.770
Skin class (T1 v T2 v T3 v T4)†	105	T1 (1/4.63)	<.001
		T2 (4/13.33)	
		T3 (11/5.66)	
		T4 (18/10.38)	
Lymph node histology (LN1/2 v LN3 v LN4)	92	LN1/2 (6/12.51)	.002
		LN3 (15/13.90)	
		LN4 (9/3.59)	
Adenopathy (palpable v not palpable)	104	Present (30/21.15)	.002
Blood involvement (smear or ERC positive)	105	CTCL (22/10.75)	<.001
Visceral disease (liver or marrow involved)	101	Present (11/4.16)	<.001

CTCL, cutaneous T cell lymphoma.

*Observed/expected is the observed number of failures in the defined group divided by the expected number of failures in the group. The expected value is calculated using the standard procedure described by Mantel,³¹ under the null hypothesis that there exists no difference between the groups.

†All pair-wise comparisons between different T categories were significant to $P < .05$ except T1 v T2 ($P = .78$) and T3 v T4 ($P = 0.75$). For multivariate analysis, the T1 and T2 patients were combined; similarly, the T3 and T4 patients were combined.

the number of circulating tumor cells also had an effect on outcome. In our series, in addition to cell size, lymphocytosis had an adverse effect on survival. This effect was most clearly seen in a survival advantage for small-cell patients with normal lymphocyte concentrations. For patients with elevated lymphocyte counts, survival was poor irrespective of cell size.

Occasional cells with electron-microscopic morphology similar to that of Sézary cells are frequently found in the skin and blood of patients with benign dermatoses.³⁶⁻³⁸ These findings are not surprising since CTCL represents a malignancy of mature helper T cells, and normal as well as reactive T cells may resemble their malignant counterpart morphologically.¹ In our series, patients with benign disorders did not have cells with the degree of the nuclear abnormalities and density of chromatin found in CTCL. No patient with a benign skin disorder and no normal individual met our criteria for CTCL cell infiltration. Occasional patients with CTCL had increased numbers of cells with minor nuclear irregularities. These patients were predominantly from advanced disease subsets. Determination of whether the lymphoid cells in these patients represent reactive cells or circulating tumor cells with a relatively bland morphology must await techniques that can clearly establish clonality and measure the extent of the infiltration. Use of DNA probes for the β T cell receptor gene has demonstrated clonal rearrangements in tissues histologically involved with CTCL.^{39,40} With the sensitivity to detect 1% to 5% tumor cells in a population, DNA probes also offer promise of detecting CTCL cells in apparently benign lymph nodes with

dermatopathic lymphadenopathy⁴⁰ and among circulating lymphocytes. In this study, E rosette cytology did not increase the sensitivity of detecting peripheral blood involvement, since only four patients had a positive ERC after a negative or suspicious smear was found. We previously reported similar results with electron microscopy,²⁸ and probably neither expensive procedure would increase the yield of positive findings. Cytogenetic analysis clearly does increase the sensitivity of detection, and flow cytometry may detect small numbers of cells when aneuploidy is present.^{13,15} The prognostic significance of detecting small numbers of such cells is unclear, however.

Detailed examination of peripheral blood smears is a noninvasive, inexpensive means of assessing extracutaneous involvement by CTCL. In nonerythrodermic CTCL, our results suggest that identification of blood involvement may be useful in identifying a small subset of patients with inferior survival while they are receiving current therapy. Because blood involvement is nearly universal in erythrodermic CTCL, examination of the peripheral blood smear can also be helpful in confirming a diagnosis of CTCL in patients with chronic erythroderma and nondiagnostic skin biopsies. In addition, assessment of the cell size distribution of the circulating CTCL cells will be useful in distinguishing patients with small-cell type whose course is relatively indolent from patients with the more aggressive mixed-cell type.

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Abnormal Prothrombin in the Plasma of Rats Carrying Hepatic Tumors

By D.V. Shah, J.A. Engelke, and J.W. Suttie

Vitamin K is required for the posttranslational formation of γ -carboxyglutamyl residues in a number of plasma clotting factors. Interference with vitamin K action results in the appearance of abnormal (des- γ -carboxy) forms of prothrombin in human plasma. Vitamin K-sufficient patients with primary hepatocellular carcinoma also secrete significant quantities of abnormal prothrombin; this response has now been studied in a rat model. Normal Buffalo strain rats had 9 $\mu\text{g/mL}$ of circulating plasma abnormal prothrombin, whereas Buffalo strain rats carrying the transplantable Morris hepatoma tumor no. 7777 had 33 $\mu\text{g/mL}$ at 3 weeks

after transplant. Vitamin K-dependent carboxylase activity was normal in the liver of these rats, but very low in the tumor tissue. Rats carrying Morris hepatoma tumors no. 9618A and 5123D did not secrete significant amounts of abnormal prothrombin. Carboxylase activity in these tumors was 15 times that of the 7777 tumor. The data suggest that the secretion of abnormal prothrombin by hepatocellular tumors is the result of normal expression of the prothrombin gene by those tumors and a failure of the tumor to express the carboxylase gene.

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VITAMIN K is required for the synthesis of clotting factors II (prothrombin), VII, IX, and X, and plasma proteins S, C, and Z.¹ The vitamin functions as a cofactor in the posttranslational carboxylation of specific glutamyl residues in microsomal protein precursors to form γ -carboxyglutamyl (Gla) residues in biologically active completed proteins.² In the absence of vitamin K or when its action is antagonized by coumarin anticoagulants, des- γ -carboxy or "abnormal" forms of prothrombin are excreted into human³ and bovine⁴ plasma. These proteins differ from prothrombin in their failure to adsorb to insoluble barium salts, to bind calcium ions in solution, or to demonstrate the Ca^{2+} -dependent phospholipid association needed for normal physiological activation.⁵ The abnormal prothrombin will, however, yield thrombin⁶ when treated with a nonphysiological activator such as the protease from *Echis carinatus* venom.⁷

A survey⁸ of the existence of abnormal prothrombin in the plasma of anticoagulant-treated species other than the human or bovine reported substantial amounts in chick, small amounts in rat and mouse, and insignificant amounts in hamster, guinea pig, rabbit, and dog plasma. The basis for the wide species variation in the excretion of abnormal prothrombin has not been determined. Development of a more sensitive amidolytic assay for rat prothrombin⁹ has shown that plasma from vitamin K-deficient or warfarin-treated rats contains a non- BaSO_4 adsorbable (undercarboxylated) pool of prothrombin that is equivalent to between 5% and 10% of the normal plasma prothrombin concentration. Conformation-specific antibodies directed toward this class of human prothrombin have been developed¹⁰ and used to

demonstrate that the concentration of abnormal prothrombin is very low in normal adults, increases substantially with oral anticoagulation or vitamin K deficiency, and is slightly increased in many patients with liver disease.¹¹

More recently, it was reported¹² that abnormal prothrombin was more drastically increased in patients with primary hepatocellular carcinoma than in other hepatic disorders and that this response was not due to vitamin K deficiency. The molecular basis for increased levels of abnormal prothrombin in diagnosed cases of hepatic carcinoma has not been established. The response has now been studied using the tumor-bearing rat as a model.

MATERIALS AND METHODS

Treatment of animals. Male rats of the Buffalo strain, weighing 50 to 60 g, were maintained on Purina laboratory chow. Vitamin K-supplemented rats were administered 1 mg of phyloquinone (Konakion, Hoffman-La Roche, Nutley, NJ) intramuscularly (IM) each week for 3 consecutive weeks. Morris hepatoma tumors no. 7777, 5123D, or 9618A were transplanted by disrupting ~1 g of tumor tissue in 5 mL of sterile Dulbecco's modified Eagle's medium (DMEM) and giving a 0.5 mL injection in each hind leg of a recipient rat.¹³

Prothrombin and abnormal prothrombin assay. Rat blood for these amidolytic assays was collected by cardiac puncture in 0.15 mol/L of potassium oxalate (10% vol/vol), centrifuged for 20 minutes at 2,000 g at 4 °C, and individual plasma samples were stored at -30 °C. Vitamin K-dependent clotting factors were adsorbed from individual plasma samples by adding 35 mg/mL of citrate-washed BaSO_4 and stirring for 1 hour at 0 °C. Prothrombin and abnormal prothrombin concentrations were determined by assay of thrombin-catalyzed amidolysis of the chromogenic peptide substrate, H-D-Phe-Pip-Arg-pna (S2238 from Kabi Diagnostics, Stockholm) as previously described.⁹ Plasma prothrombin (normal) was measured with the chromogenic substrate after activation with a calf serum factor mix⁹ and commercial thromboplastin (Thromboplastin, Sigma Diagnostics, St Louis), and total normal plus abnormal prothrombin was measured after activation with *E carinatus* venom. Abnormal prothrombin was measured by activation of the BaSO_4 -adsorbed plasma with *E carinatus* venom (Sigma).

Vitamin K-dependent carboxylation. Buffalo strain rats carrying the transplantable Morris hepatoma tumors were used when they weighed 160 to 175 g. When administered, sodium warfarin (5 mg/kg) was given by intraperitoneal (IP) injection 18 hours prior to killing. Holtzman strain rats were used as normal controls in carboxylase assay so that the enzyme activities from different rat strains could be standardized. Rats were killed, and a crude microsomal preparation of both liver and tumor tissue was prepared as previously described,¹⁴ except that rats were not fasted. A Triton

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X-100 (RPI, Elk Grove Village, IL) solubilized preparation of microsomes equivalent to 0.5 g of liver or 1.0 g of excised tumor mass per milliliter were obtained and incubated at 17 °C for 30 minutes to determine the vitamin K-dependent incorporation of $H^{14}CO_3$ into the substrate Boc-Glu-Glu-Leu-OMe (BaChem Fine Chemicals, Torrance, CA).^{15,16}

RESULTS

Absorption with $BaSO_4$ is capable of removing all but a small fraction of prothrombin in the plasma of vitamin K-sufficient rats.⁹ When non- $BaSO_4$ adsorbable prothrombin (abnormal prothrombin) was measured in the plasma of Buffalo strain rats carrying three different Morris hepatoma tumors, a significant increase was seen in the rats carrying the most rapidly growing tumor (Table 1). Buffalo rats carrying the transplantable Morris hepatoma tumor no. 7777 had an approximate fourfold increase in circulating abnormal prothrombin as compared with normal Buffalo rats. Partially carboxylated forms of prothrombin are present following anticoagulation,^{17,18} and these forms differ in their ability to adsorb to barium salts.¹⁹ $BaSO_4$ treatment removed 97% of the thromboplastin-activatable prothrombin in normal plasma but only 85% of this prothrombin species in the plasma of tumor-bearing Buffalo strain rats. These data suggest that a significant amount of partially carboxylated prothrombin is also secreted in tumor-bearing Buffalo strain rats. The concentration of plasma abnormal prothrombin was very much dependent on the period of time after tumor transfer. As shown in Fig 1, normal Buffalo strain rats had 8 to 9 $\mu g/mL$ of circulating plasma abnormal prothrombin, whereas Buffalo strain rats carrying the transplantable Morris hepatoma tumor no. 7777 had 10 $\mu g/mL$ at 2 weeks and 33 $\mu g/mL$ at 3 weeks after transplant. High concentrations of plasma abnormal prothrombin were noted only near the end of the transplant period and presumably reflected tumor mass.

In an attempt to discover the cause of the abnormal prothrombin secretion in tumor-bearing rats, liver and tumor tissue vitamin K-dependent carboxylase activities were determined. Carboxylase activity was slightly higher in the livers of Buffalo strain rats carrying the transplantable Morris hepatoma tumor no. 7777 as compared with normal Buffalo strain rats (Fig 2), but the activity of this enzyme in tumor tissue was <5% of that in normal liver tissue. Liver vitamin K-dependent carboxylase activity is increased by a

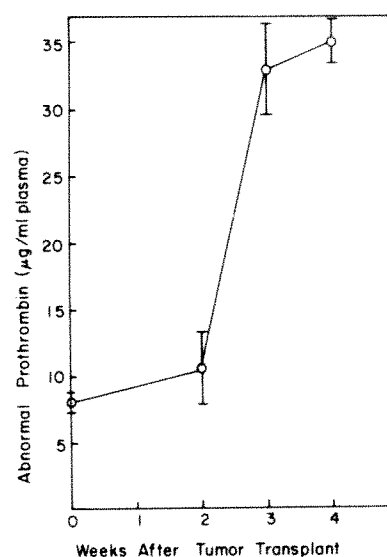


Fig 1. Appearance of abnormal prothrombin after transplant of Morris hepatoma tumor no. 7777. Plasma samples from each rat were assayed after $BaSO_4$ adsorption as described in the Materials and Methods section. Values are mean $\pm$ SEM for three to six rats per group.

vitamin K deficiency,¹⁴ and carboxylase activity in both normal and tumor-bearing rats and in tumor tissue was depressed when the rats were supplemented with vitamin K (Fig 2). Vitamin K-supplemented Buffalo rats carrying the transplantable Morris hepatoma tumor no. 7777 had the same fourfold increase in circulating abnormal prothrombin as compared with K-supplemented normal Buffalo rats (data not shown) as is shown in Table 1, indicating that the elevated levels of abnormal prothrombin in circulation were not due to vitamin K deficiency.

The response of normal rats to vitamin K deficiency or warfarin administration is an increase in the activity of the liver microsomal vitamin K-dependent carboxylase¹⁴ and an increase in the concentration of microsomal prothrombin precursors.^{8,20} The livers of rats carrying the no. 7777 tumor demonstrated the same responses, but warfarin administration had no influence on vitamin K-dependent carboxylase activity or microsomal prothrombin precursor concentration of the tumor tissue (Figs 3 and 4).

Rats carrying transplantable Morris hepatoma tumors no.

Table 1. Plasma Normal and Abnormal Prothrombin Concentrations in Tumor-Carrying Buffalo Rats

Tumor No.	Weeks Between Transfer	Plasma Prothrombin Concentrations ($\mu g/mL$)			
		<i>Echis carinatus</i> Activation		Thromboplastin Activation	
		Before $BaSO_4$	After $BaSO_4$ *	Before $BaSO_4$ †	After $BaSO_4$
No tumor (12)	—	103 $\pm$ 3	9 $\pm$ 1	98 $\pm$ 2	3 $\pm$ 1
7777 (9)	3–4	113 $\pm$ 2	34 $\pm$ 4‡	100 $\pm$ 2	15 $\pm$ 2
5123D (12)	6–8	110 $\pm$ 3	12 $\pm$ 1	112 $\pm$ 3	11 $\pm$ 1
9618A (9)	8–9	119 $\pm$ 3	11 $\pm$ 1	110 $\pm$ 4	5 $\pm$ 1

Plasma for analysis was obtained at the time of tumor transfer. The three different tumor strains have different growth rates, and were transferred when the mass of the tumors was similar. Values are mean $\pm$ SEM for the number of animals indicated in parentheses.

*"Abnormal" prothrombin.

†"Normal" prothrombin.

‡Significantly higher ($P < .01$) than the other three treatments.

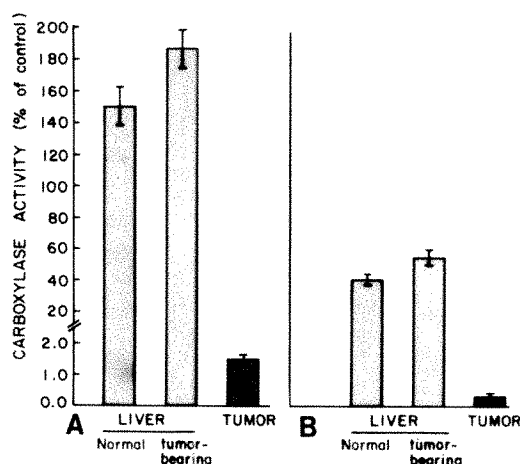


Fig 2. Vitamin K-dependent carboxylase activity in liver and tumor tissue of rats. Normal and tumor-bearing Buffalo strain rats were killed 3 weeks after transplant of Morris hepatoma tumor no. 7777. The rats were fed a commercial laboratory chow diet and administered either 1 mg of phyloquinone or saline each week. Vitamin K-dependent carboxylase activity was determined as described in the Materials and Methods section. Data were obtained on different days, and the carboxylase activity of two vitamin K-sufficient Holtzman strain rats assayed each day was used as the 100% control activity. Values are mean $\pm$ SEM for 6 to 18 rats in each group. (A) Chow diet only; (B) vitamin K supplement.

5123D and 9618A did not secrete significant amounts of abnormal prothrombin (Table 1). Vitamin K-dependent carboxylase activity and microsomal prothrombin precursor concentration in the livers of normal Buffalo strain rats and the livers of these rats carrying transplantable Morris hepatoma tumors no. 5123D and 9618A were compared. Similar liver carboxylase activities and microsomal precursor con-

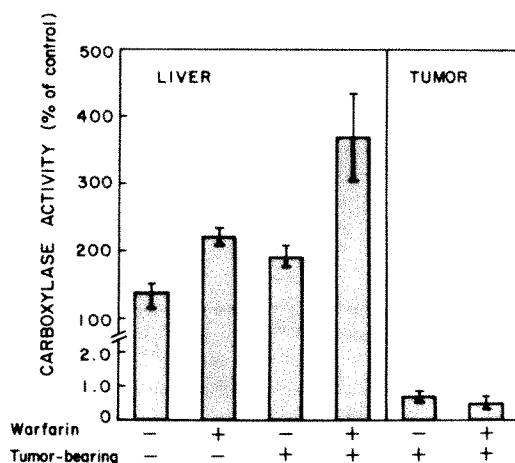


Fig 3. Effect of warfarin administration on microsomal vitamin K-dependent carboxylase activity in liver and tumor tissue. Normal rats and rats carrying a Morris hepatoma tumor no. 7777 for 3 weeks were administered either warfarin (5 mg/kg, intraperitoneally) or saline 18 hours before being killed. Vitamin K-dependent carboxylase activity was determined as described in the Materials and Methods section. Carboxylase activity of four normal Holtzman strain rats was used as the 100% control activity. Values are mean $\pm$ SEM for three to four rats in each group.

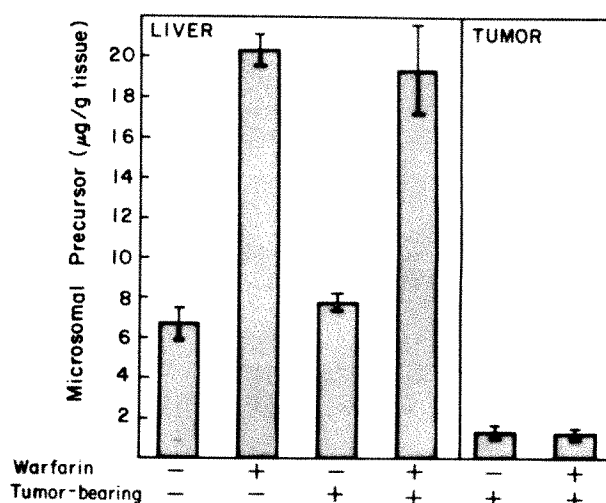


Fig 4. Effect of warfarin administration on microsomal prothrombin precursor concentration in liver and tumor tissue. Microsomal precursor concentration was determined as described in the Materials and Methods section. Other conditions were as described in the legend to Fig 3.

centrations were found (Fig 5) in rats carrying these two tumors. Activity of the vitamin K-dependent carboxylase in the tumor tissue from these rats was, however, ~20% to 30% of that in the host rat liver (Fig 5). This activity was ~15 times higher than the activity observed in the no. 7777 tumor tissue shown in Fig 2. The microsomal prothrombin precursor concentration in the liver of the rats carrying the three

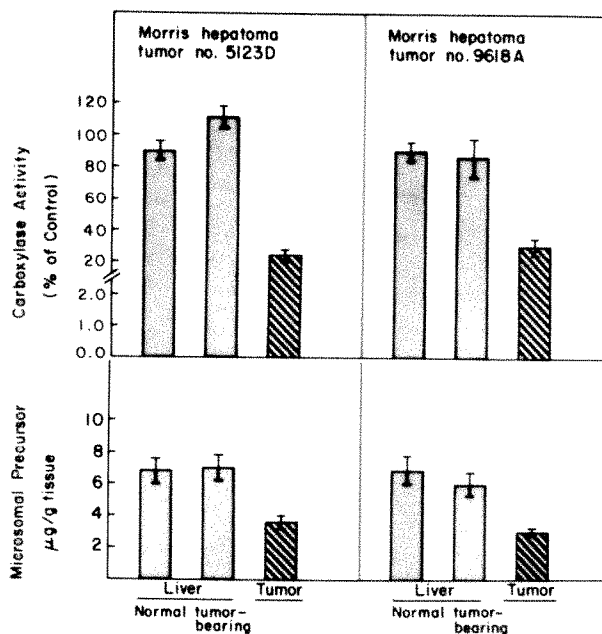


Fig 5. Vitamin K-dependent carboxylase activity and microsomal prothrombin precursor concentrations in liver and tumor tissue of rats. Normal and tumor-bearing rats were killed 6 to 8 weeks or 8 to 9 weeks after transplantation of Morris hepatoma tumor no. 5123D or 9618A. Assays were as described in Figs 3 and 4. Data are from a single experiment representative of three experiments. Values are mean $\pm$ SEM for three to six rats in each group.

different Morris hepatoma tumors was, however, comparable (Figs 4 and 5), and liver vitamin K-dependent carboxylase activity was somewhat higher in rats carrying the no. 7777 tumor as compared with the 5123D or 9618A tumor (compare Figs 3 and 5).

DISCUSSION

Liebman and colleagues¹² detected elevated abnormal prothrombin in the serum of patients with biopsy-confirmed hepatocellular carcinoma and established that this elevation does not reflect a vitamin K deficiency in these patients. The data of the present study demonstrate that the rat is a suitable model for studying this response. Adsorption of oxalated plasma with BaSO₄ will remove almost all normal prothrombin,⁹ and increases in venom-generated thrombin above the background represent secretion of abnormal prothrombin. The plasma of rats carrying one of three strains of Morris hepatoma tumors contained significant amounts of abnormal prothrombin.

These data are consistent with the hypothesis that the tumors that increase circulating abnormal prothrombin are those that are capable of expressing the prothrombin gene but that have lost the ability to express significant levels of the vitamin K-dependent carboxylase enzyme. This view is supported by the low concentrations of this enzyme measured in the no. 7777 tumor tissue as compared with much higher concentrations in the 5123D and 9618A lines, which secreted less abnormal prothrombin. It is also possible that the no. 7777 tumor line contains an inhibitor of the enzyme

or that turnover rate of the enzyme has been substantially increased. The failure of the no. 7777 tissue to increase carboxylase activity or microsomal prothrombin precursor concentrations following warfarin administration, however, also suggests a low level of expression of this gene. Normal rat liver responds to warfarin administration with an increase in both precursor concentration and enzyme activity^{14,20} and is evidence^{14,21,22} that association of the precursor with the enzyme is responsible for these changes. The delay in abnormal prothrombin appearance following transfer of the Morris hepatoma tumors is consistent with the need for a significant tumor mass to develop before this product is seen in plasma.

Vitamin K-dependent carboxylase activity was consistently elevated in the liver of rats carrying tumors. This may reflect the response of carboxylation activity to des- γ -carboxy forms of vitamin K-dependent clotting factors or other unknown factors present in plasma of hypoprothrombinemic animals.²³⁻²⁷ Availability of rat hepatoma lines that do secrete large amounts of abnormal prothrombin in the absence of carboxylation provides a valuable tool with which to study this response.

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Chromosome Abnormalities in AIDS-Associated Lymphadenopathy

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Cytogenetic studies were performed on direct and 24-hour culture preparations of eight lymph node biopsies from seven patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC)-associated lymphadenopathy in whom histological evidence of lymphoma was not detected. Three of these seven had chromosomal abnormalities, including chromosome instability in one and clonal chromosomal abnormalities in two; one of the latter was a t(8;14)(q24;q32). The remaining five showed normal karyotypes. Epstein-Barr virus (EBV) titers were elevated in all three patients that exhibited chromosome abnormali-

ties, two of whom later developed malignant lymphoma. A control group of five patients with reactive lymphadenopathy not associated with AIDS failed to reveal chromosomal aberrations, but elevated EBV titers were present in two. These data are consistent with current views on the role of EBV and chromosome change in the development of lymphoma in immunodeficient states and suggest that karyotypically abnormal AIDS-related lymphadenopathy represents a prelymphomatous proliferation.

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THE ACQUIRED immunodeficiency syndrome (AIDS) comprises a group of clinical presentations that have recently emerged as an epidemic among subgroups of homosexual males, intravenous (IV) drug users, blood transfusion recipients, and others. *Pneumocystis carinii*, cytomegalovirus (CMV) and other opportunistic infections, autoimmune phenomena, generalized lymphadenopathy, lymphoma, and Kaposi's sarcoma (KS) have characterized this disorder.¹⁻⁵ A prodromal phase consisting of fever, weight loss, and lymphadenopathy frequently precedes opportunistic infections. Lymphopenia, cutaneous anergy, decreased in vitro responsiveness to mitogens, decreased T helper cells, and inversion of the T helper/T suppressor ratio are common immunological abnormalities in AIDS.⁶⁻⁸ The human T cell lymphotropic virus (HTLV-III) has recently been shown to play an etiologic role in AIDS.⁹⁻¹² Currently, patients in high-risk groups for AIDS with generalized lymphadenopathy but lacking evidence of KS or opportunistic infection are classified as having the AIDS-related complex (ARC). The types of neoplasms seen in this disorder are similar to those seen in posttransplantation immunosuppressed patients^{13,14} and include a Burkitt's-like undifferentiated lymphoma with characteristic rearrangements involving chromosomes 8, 14, and 22 and a probable Epstein-Barr virus (EBV)-related etiology.¹⁵⁻¹⁷ Although current evidence suggests that the lymphomas in immunosuppressed states arise among polyclonal lymphoid proliferations by selective expansion of monoclonal cells characterized by specific chromosome rearrangements, the cytogenetic nature of the prelymphomatous proliferations is essentially unknown. To address this question, we performed cytogenetic analysis of lymph node biopsies from seven patients with generalized lymphadenopathy arising in a clinical spectrum of AIDS (two cases) or ARC (five cases). For comparison, we also studied a control group of five patients with reactive lymphadenopathy not associated with AIDS or ARC. Our results show that chromosome instability and clonal changes characterize only AIDS or ARC-associated proliferations.

MATERIALS AND METHODS

All patients had lymphadenopathy, which was generalized in the seven patients with AIDS or ARC (group I, Table 1) and localized in five who did have AIDS or ARC (group II). Lymph node histology and laboratory data of all patients studied are summarized in Table 1. All seven group I patients had evidence of an immune dysfunction

based on markedly depressed lymphoproliferative responses to a variety of mitogens and antigens. All group I cases were in high-risk groups for AIDS. Those classified as having AIDS had KS or an opportunistic infection, and those classified as having ARC did not. Chromosome preparations were made from single-cell suspensions of freshly obtained lymph node biopsies. The cells were incubated 1 to 3 hours in colcemid (final concentration of 0.1 µg/mL) or were cultured for 24 hours in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS) and the antibiotic penicillin-streptomycin. Colcemid was added to the cultures during the final hour. Cell harvest and chromosome preparation were made following conventional methods using 0.075 mol/L of potassium chloride as the hypotonic solution and methanol/acetic acid 3:1 as the fixative. Karyotyping was performed from trypsin-Giemsa or quinacrine-banded preparations. All histologic specimens were reviewed, and lymphomas were classified using the Working Formulation.¹⁸

RESULTS

Of the 13 nodes studied, three showed chromosomal abnormalities [patients 1 (first biopsy), 3, and 6—group I, Table 1]. Biopsies from patients 1 and 3 exhibited mixtures of normal and clonally abnormal cells. In biopsy 1 of patient 1, of the 30 cells analyzed, 27 were normal (46,XY or hypodiploid due to random loss of chromosomes) and three had rearranged chromosomes. The chromosomal complements of the latter were the following respectively: (a) 46,XY,t(8;14)(q24;q32), (b) 46,X,-Y,t(8;14)(q24;q32), +mar1, and (c) 43,X,-Y,-8,-14,-16,-19,+8q,-, +mar1. The mar1 chromosome was comprised of the entire long arm of

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Table 1. Clinical Features and Results of Laboratory and Cytogenetic Evaluation of Patients Having Lymphadenopathy, Either in Association With (Group I) or Lacking (Group II) AIDS or ARC

Patient	Age/Sex	Biopsy Site	Histology	CMV Titers	EBV Titers	Cytogenetics
						No. of Cells/Karyotype
Group I, lymphadenopathy associated with AIDS or ARC						
1 (AIDS)	38/M	Left epitrochlear lymph node	Marked follicular hyperplasia with pericapsular small foci of KS	1:64	1:2560	24: 46,XY 3: 45,XY* 1: 46,XY,t(8;14)(q24;q32), + mar 1 1: 46,X,-Y,t(8;14)(q24;q32), + mar 1 1: 43,X,-7,-8,-14,-16,-19,+8q-, + mar 1 64: 46,XY
		Right cervical lymph node	Marked follicular hyperplasia with small foci of KS			
2 (ARC)	26/M	Left cervical lymph node	Marked follicular hyperplasia	ND	1:160	14: 46,XY 2: 45,XY* 1: 44,XY*
3 (ARC)	33/M	Right cervical lymph node	Marked follicular hyperplasia	ND	1:320	14: 46,XY 3: 44,XY* 3: 47,XY, + mar 1
4 (ARC)	26/M	Right cervical lymph node	Marked follicular hyperplasia	ND	1:1280	16: 46,XY 2: 45,XY*
5 (ARC)	30/M	Right cervical lymph node	Marked follicular hyperplasia	1:64	ND	20: 46,XY
6 (ARC)	32/M	Left axillary lymph node	Marked follicular hyperplasia	1:128	1:5120	9: 46,XY 1: 45,XY* 3: 44,XY* 1: Chromosome breakage
7 (AIDS)	31/M	Right cervical lymph node	Marked follicular hyperplasia	1:256	1:10,240	25: 46,XY
Group II, lymphadenopathy not associated with AIDS						
8	17/M	Right cervical lymph node	Follicular hyperplasia with few clusters of epithelioid cells	ND	ND	25: 46,XY
9	62/F	Left axillary lymph node	Follicular hyperplasia	ND	ND	25: 46,XX
10	58/M	Right cervical lymph node	Follicular hyperplasia with few clusters of epithelioid cells	ND	1:1280	25: 46,XY
11	33/F	Left cervical lymph node	Follicular hyperplasia	ND	ND	25: 46,XY
12	31/F	Left submandibular lymph node	Follicular hyperplasia	ND	1:1280	25: 46,XX

ARC, AIDS-related complex; CMV, cytomegalovirus; EBV, Epstein-Barr virus; KS, Kaposi's sarcoma; ND, not done.

*Hypodiploid chromosome numbers represent random chromosome loss due to cell rupture during preparation.

chromosome 1. Thus, the presence of the 8q- and mar1 chromosomes in the third cell indicates that it also belongs to the same abnormal clone as the other two cells. The absence of the 14q+ chromosome in it probably represents random loss of this chromosome, only a single copy of chromosome 14 being present in this cell. In the biopsy from patient 3 (Table 1), of 20 cells analyzed, 18 were normal (46,XY or hypodiploid due to random loss of chromosomes) and two had a small acrocentric marker chromosome in addition to an otherwise normal chromosome complement. The two cells with the marker chromosome thus identify a clonal proliferation with the karyotype 47,XY,+mar1. The derivation of mar1 in these cells could not be determined due to its nonspecific banding pattern. Among the cells analyzed from the biopsy of patient 6, nonspecific chromosome and chroma-

tid breakage was seen in 3 of 16 (19%) (Table 1). The remaining ten nodes including five each from groups I and II revealed only normal chromosomal complements (46,XY or hypodiploid due to random loss of chromosomes).

EBV titers, shown in Table 1, were elevated in two group II patients with negative monospot tests. No chromosome abnormalities were present in biopsies from these patients. EBV titers were elevated in six of the seven group I patients. The patient with the highest EBV titer (patient 7, group I, Table 1) lacked cytogenetic aberrations. A subsequent non-Hodgkin's lymphoma developed in patients 1, 4, and 6. The lymphoma in patient 1 was classified as immunoblastic B cell type, and in patient 4 was classified as diffuse large cell type. Histologic slides showing lymphoma in case 6 were unavailable for review for classification.

DISCUSSION

The pattern of lymphoma development in AIDS is strikingly similar to that in two other immunodeficiency states, namely, posttransplantation immunodepression and X-linked lymphoproliferative syndrome.¹³ Lymphomagenesis in both disorders is increasingly recognized to be linked to EBV infection.¹⁹ In these cases, a polyclonal proliferation of B cells, probably initiated by viral infection, is suggested to lead to monoclonal expansion of malignant cells characterized by specific chromosome rearrangements that bring about juxtaposition of the *c-myc* oncogene and the immunoglobulin loci, resulting in *c-myc* deregulation.²⁰⁻²² Such a model implies that the proliferating polyclonal cells which may be preneoplastic are genetically unstable and exhibit chromosome rearrangements that include the abovementioned lymphoma-specific ones.²³ Cytogenetic data pertaining to this proliferation have not been available until now.

In our study, three of eight reactive lymph nodes associated with AIDS or ARC (group I) revealed chromosome abnormalities. All three patients, two of whom later developed lymphoma also had high EBV titers. Another group I patient with high EBV titers eventually developed a non-

Hodgkin's lymphoma, and two group II patients with high titers remain alive and well 12 to 19 months following diagnosis. We did not detect a chromosome abnormality in the lymph node biopsies from these latter three patients, suggesting that EBV infection may precede the appearance of cytogenetic abnormalities. Unfortunately, tissues from lymphomas were not available to us for cytogenetic analysis. In contrast, five reactive lymphadenopathies unassociated with AIDS or ARC (group II) failed to reveal chromosome aberrations despite elevated EBV titers in two.

The cytogenetic evidence presented here strongly suggest that the lymphadenopathies of AIDS or ARC patients represent prelymphomatous proliferations, which is in accord with histopathological evidence for the development of non-Hodgkin's lymphoma correlated with AIDS lymphadenopathy presented by other investigators.^{14,24-27} EBV infection may be related to the chromosome breakage, which precedes lymphoma development. Further cytogenetic and molecular studies of AIDS-related lymphadenopathy should clarify the significance of this proliferation to lymphoma development.

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Sodium Fluoride Mimics Effects of Both Agonists and Antagonists on Intact Human Platelets by Simultaneous Modulation of Phospholipase C and Adenylate Cyclase Activity

By Joachim Kienast, Jef Arnout, György Pfliegler, Hans Deckmyn, Bernard Hoet, and Jos Vermynen

Using intact human platelets, we studied the effect of sodium fluoride (NaF) on platelet aggregation and release reaction and correlated the functional changes to intracellular events specific for either agonist-induced or antagonist-induced platelet responses. At lower concentrations, with a peak activity between 30 and 40 mmol/L, NaF induced aggregation and release of adenosine 5'-triphosphate (ATP) that was associated with increased formation of inositol phosphates, a rise in cytosolic free Ca^{2+} , and phosphorylation of 20-kd and 40-kd proteins. At NaF concentrations >40 mmol/L, aggregation and ATP release decreased dose-dependently in parallel with a decrease in Ca^{2+} mobilization, whereas neither inositol phosphate formation nor 40-kd protein phosphorylation was reduced. At these concentrations, NaF caused a dose-dependent transient rise in platelet cyclic adenosine 3',5'-monophosphate (cAMP) levels that was sufficient to account for the

observed reduction in Ca^{2+} mobilization, aggregation, and ATP release. Stimulated cAMP levels started declining rapidly within 30 seconds of addition of NaF, however. Similarly, prostacyclin (PGI_2)-induced cAMP accumulation was temporarily enhanced but subsequently suppressed by NaF, suggesting either stimulation of a cAMP phosphodiesterase or delayed inhibition of adenylate cyclase. Evidence for the latter was provided by the finding that NaF pretreatment of platelets resulted in partial inhibition of PGI_2 -stimulated cAMP formation in the presence of the cAMP phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (MIX). We conclude that NaF exerts a dual (stimulatory and inhibitory) effect on adenylate cyclase in intact platelets that is accompanied by simultaneous activation of a phosphoinositide-specific phospholipase C; in addition, a cAMP phosphodiesterase may be activated.
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PLATELETS REACT to a variety of biologically active substances (agonists) with partial or full activation characterized by shape change, release of granule contents, and aggregation. Conversely, other agents (antagonists) such as prostacyclin (PGI_2) or prostaglandin E_1 induce a state of reduced responsiveness to agonists.^{1,2} The nature of a platelet response initiated by receptor occupancy depends on the selective coupling of a particular receptor to at least two major messenger-generating systems. One involves phospholipase C-catalyzed hydrolysis of membrane inositol phospholipids, Ca^{2+} mobilization, activation of protein kinase C, and arachidonic acid liberation, whereas the other is related to the formation of cyclic adenosine 3',5' monophosphate (cAMP) catalyzed by adenylate cyclase.^{3,4} Adenylate cyclase activity is subject to regulation by two distinct guanine nucleotide-binding proteins (also referred to as G or N proteins) that couple the enzyme to specific membrane receptors and allow receptor expression as either N_s -mediated stimulation of N_i -mediated inhibition of its catalytic subunit.⁵⁻⁷ In addition, more recent evidence gave rise to the hypothesis that a guanine nucleotide-binding protein might also constitute the link that couples stimulatory platelet receptors to phospholipase C activation and/or Ca^{2+} mobilization.⁸⁻¹⁰

Fluoride activates N proteins by promoting the dissociation of their subunits.^{5,11-13} In isolated platelet membranes, sodium fluoride (NaF) both stimulates and inhibits adenylate cyclase through activation of N_s and N_i respectively.^{12,13} Conflicting results have been reported regarding the influence of NaF on intact platelets. Enhanced cAMP formation has been described¹⁴ as have NaF-induced mobilization of cytosolic free Ca^{2+} , platelet aggregation, and serotonin release.¹⁵⁻¹⁷ These findings led us to investigate the effects of NaF on platelet function further, with particular attention to intracellular regulatory processes. In this article we demonstrate that NaF has a concentration-dependent, biphasic effect on platelet activation as a result of simultaneous modulation of phospholipase C and adenylate cyclase

activity. We suggest that NaF-sensitive N proteins are not only involved in the regulation of adenylate cyclase but may also play a role in phospholipase C activation.

MATERIALS AND METHODS

Reagents. [2,8- ^3H]Adenine (17.7 Ci/mmol) and [8- ^{14}C] cAMP phosphate (45.1 mCi/mmol) were obtained from New England Nuclear (Boston). Carrier-free [^{32}P]orthophosphate (40 mCi/mL in aqueous solution), myo-[2- ^3H]inositol (13.8 Ci/mmol), and quin 2 acetoxymethyl tetraester (Quin 2/AM) came from Amersham International (Amersham, England). Insta-Gel II was from Packard Instrument B.V. Chemical Operations (Groningen, The Netherlands). AG 50W-X4 cation exchange resin (200 to 400 mesh) was obtained from Bio-Rad Laboratories (Richmond, CA), and Dowex 1x8 anion exchange resin (200 to 400 mesh) was obtained from Fluka (Buchs, Switzerland). Sepharose 2B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Apyrase, arachidonic acid and adenosine 5'-diphosphate came from Sigma Chemical (St Louis) and 3-isobutyl-1-methyl-xanthine (MIX) from Janssen Chimica (Beerse, Belgium). Platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) was from Boehringer (Mann-

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heim, FRG), A23187 from Calbiochem-Behring Corp (San Diego), bovine thrombin from Roche (Brussels, Belgium), and collagen from Hormon-Chemie (Munich, FRG). PGI₂ was obtained from Upjohn (Kalamazoo, MI). Luciferin/luciferase reagent came from Chrono-Log Corp (Havertown, PA) and NaF from Baker Chemical (Phillipsburg, NJ). Bovine serum albumin (BSA) was from Organon Teknika (Boxtel, the Netherlands). CV-3988 (rac-3-(*N*-octadecyl-carbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate) was kindly provided by Takeda Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade obtained from various sources.

Preparation of platelet-rich plasma. Blood was obtained by clean venipuncture of healthy volunteers who denied having taken any drugs during the previous 2 weeks. Anticoagulants used were either 0.1 vol of 106 mmol/L of trisodium citrate, 0.03 vol of 0.3 mol/L of EDTA, or 0.14 vol of ACD buffer (85 mmol/L of trisodium citrate, 71 mmol/L of citric acid, 111 mmol/L of dextrose, pH 4.5) as specified below. Platelet-rich plasma (PRP) was prepared by centrifugation at 150 g for 10 minutes at 20 °C.

Stimulation of platelets by NaF. For stimulation of platelets by NaF, 0.1 vol of prepared reaction mixtures containing NaF in distilled water in the appropriate amount to yield final concentrations of NaF between 10 and 100 mmol/L as indicated was added to 0.9 vol of platelet suspensions. To preclude any influence by Na⁺ or by changes in osmolarity, the reaction mixtures were made up with NaCl to give 1-mol/L solutions; 0.1 vol of 1 mol/L of NaCl in distilled water was added to controls. These principles were retained throughout all experimental procedures.

Platelet aggregation and ATP release. Aliquots of 0.45 mL of citrated PRP together with 0.05 mL of reconstituted luciferin/luciferase reagent (40 mg in 1 mL of sterile water) were placed into aggregometer cuvettes and preincubated while being stirred for 1 minute at 37 °C in a Lumi-Aggregometer Model 400 (Chrono-Log, Havertown, PA). Then 0.055 mL of NaF/NaCl-containing reaction mixtures was added. Aggregation and adenosine triphosphate (ATP) release were monitored over 10 minutes by parallel recording of light transmission and luminescence displayed on a dual-channel chart recorder. The luminescence signal was calibrated by addition of a known amount of ATP at the end of each experiment. Part of the aggregation studies was performed in an Elvi 840 dual-channel aggregometer (Elvi Logos, Milan). Threshold aggregating concentrations were defined as minimal concentrations of inducers that caused irreversible platelet aggregation within 2 minutes of addition to stirred PRP. Inhibitors (apyrase, acetylsalicylic acid, CV-3988) were added 3 minutes prior to platelet stimulation.

Measurement of [³H]inositol phosphates. NaF-induced changes in inositol phosphate formation were measured as described, with minor modifications.^{18,19} PRP obtained from 100 mL of freshly drawn blood anticoagulated with EDTA was centrifuged at 800 g for 10 minutes at 20 °C. The platelet pellet was resuspended in 3 mL of a modified Tyrode's solution-HEPES buffer (134 mmol/L of NaCl, 12 mmol/L of NaHCO₃, 2.9 mmol/L of KCl, 0.34 mmol/L of Na₂HPO₄, 5 mmol/L of HEPES, 5 mmol/L of glucose, and 1 mmol/L of EGTA pH 6.8). For labeling, the platelet suspension was incubated with myo-[2-³H]inositol (66 μCi/mL) for 3 hours at 37 °C. Platelets were then washed in 30 mL of buffer without EGTA and finally resuspended in modified Tyrode's solution-HEPES buffer (pH 7.4 at 37 °C) containing 1 mmol/L of CaCl₂ and 1 mmol/L of MgCl₂ instead of EGTA. LiCl 10 mmol/L was added to inhibit the degradation of inositol 1-phosphate to free inositol.²⁰ The final platelet concentration was adjusted to 8 × 10⁸/mL. Samples of 0.5 mL were transferred into aggregometer cuvettes and preincubated while being stirred at 37 °C for 1 minute. Then 0.055 mL of NaF/NaCl-containing reaction mixtures was added, and the incubation was continued for varying times as indicated. The reaction

was stopped by transferring the samples into 1.88 mL of chloroform/methanol/HCl (100:200:2, vol/vol). For phase separation of the extract, 0.62 mL of chloroform and 0.62 mL of water were added. After thorough mixing, the samples were centrifuged at 800 g for 10 minutes. Then 1.5 mL of the upper phase containing the water-soluble inositol phosphates was diluted in 2.5 mL of water and applied to Dowex 1 anion-exchange columns (formate form). The inositol phosphates were separated by sequential elution as described.^{18,19} Radioactivity in portions of each fraction was determined by scintillation counting in the gel phase using Insta-Gel II as scintillation fluid. Results were corrected for all loss factors.

Measurement of protein phosphorylation. Protein phosphorylation in response to stimulation of platelets by NaF was measured as ³²P incorporation into proteins after labeling of the platelet metabolic phosphate pool with carrier-free ³²PO₄.^{21,22} ACD-anticoagulated PRP was applied to Sepharose 2B columns, and platelets were eluted with Ca²⁺-free and PO₄³⁻-free Tyrode's solution (136 mmol/L of NaCl, 2.7 mmol/L of KCl, 12 mmol/L of NaHCO₃, 2 mmol/L of MgCl₂, 5.5 mmol/L of glucose pH 6.8) containing BSA (3.5 mg/mL) and apyrase (60 μg/mL). For labeling, gel-filtered platelets (3 × 10⁸/mL) were incubated with ³²PO₄ (0.8 mCi/mL) for 1 hour at 37 °C. Thereafter, platelets were again gel-filtered using Ca²⁺-restored and PO₄³⁻-restored Tyrode's solution without albumin and apyrase for elution. The platelet count was adjusted to ~2 × 10⁸/mL. Samples of 0.9 mL of the final platelet suspension were incubated while being stirred with 0.1 mL of NaF/NaCl-containing reaction mixtures at 37 °C for varying times. The incubation was terminated by addition of 0.1 mL of 3 mol/L of HClO₄. The precipitated proteins were isolated by centrifugation at 10,000 g for 5 minutes and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli buffer system.²³ Gels were stained with Coomassie brilliant blue, and the specific areas corresponding to the 40-kd and 20-kd proteins were excised and counted for Cerenkov radiation in a liquid scintillation counter.

Measurement of cytosolic free Ca²⁺ concentrations. Platelets were isolated from citrated PRP by gel filtration on Sepharose 2B using an elution buffer containing 10 mmol/L of HEPES, 145 mmol/L of NaCl, 5 mmol/L of KCl, 0.5 mmol/L of NaH₂PO₄, and 6 mmol/L of glucose pH 7.4. For loading with Quin 2, gel-filtered platelets (~1.5 × 10⁸/mL) were incubated with 10 μmol/L of Quin 2/AM for 1 hour at 37 °C. To remove extraneous dye, platelets were again gel-filtered with the same elution buffer, which contained no added Ca²⁺ or Mg²⁺ in order to prevent disturbance of the fluorescence measurements due to formation of insoluble CaF₂ or MgF₂. The final platelet count was adjusted to ~1 × 10⁸/mL. NaF-induced changes in cytosolic free Ca²⁺ concentrations ([Ca²⁺]_i) were measured as changes in Quin 2 fluorescence intensity after addition of 0.1 vol of NaF/NaCl-containing reaction mixtures in a fluorescence spectrophotometer (Locarte, England). Measurements were made at 37 °C with an excitation wavelength of 339 nm and an emission wavelength of 492 nm. Under these conditions, no autofluorescence of NaF was observed. The fluorescence signal was calibrated in separate samples in the absence of NaF essentially as described.²⁴⁻²⁶ Changes in [Ca²⁺]_i were expressed in percentage of basal [Ca²⁺]_i that was measured in resting platelets prior to addition of NaF.

Measurement of [³H]cAMP. For labeling, PRP anticoagulated with EDTA was incubated with [³H]adenine (15 μCi/mL) for 30 minutes at 20 °C. Labeling was terminated by centrifugation at 800 g for 10 minutes at 20 °C and resuspension of the platelet pellet in an equal amount of citrated autologous plasma. Aliquots of 0.45 mL of the final platelet suspension were incubated with 0.05 mL of NaF/NaCl-containing reaction mixtures at 37 °C for varying times. In some experiments, 10 mmol/L of MIX or 0.05 μmol/L of PGI₂ was added to the platelet suspension prior to or following the

stimulation by NaF as indicated. The incubation was stopped by addition of 0.05 mL of 3 mol/L of trichloroacetic acid containing [^{14}C]cAMP (0.2 $\mu\text{Ci/mL}$) for assessment of recovery. After thorough mixing, the precipitated proteins were removed by centrifugation at 10,000 g for 5 minutes, and total ^3H - and ^{14}C -radioactivity in the supernatant was determined by liquid scintillation counting. cAMP was isolated from the trichloroacetic acid extract by ion-exchange chromatography on Dowex AG 50W-X4 followed by $\text{ZnSO}_4/\text{Ba}(\text{OH})_2$ precipitation of adenine nucleotides other than cAMP as described.^{27,28} The precipitate was spun down by centrifugation at 10,000 g for 5 minutes and ^3H - and ^{14}C -radioactivity in the final supernatant was measured. Results were corrected for spillover and loss factors and expressed as percentage of basal [^3H]adenine incorporation into cAMP that was measured under control conditions (addition of 0.1 vol of 1 mol/L of NaCl). Basal [^3H]adenine incorporation into cAMP ranged between 0.06% and 0.12%.

Measurement of total lactate dehydrogenase (LDH). Total LDH activity was measured spectrophotometrically as described.²⁹ LDH activity was determined in citrated platelet-poor plasma (PPP) either after addition of 0.1 vol of 1 mol/L of NaCl or NaF to PPP or after incubation of PRP with 0.1 vol of 1 mol/L of NaCl or NaF for 10 minutes at 37 °C and subsequent removal of platelets by centrifugation at 10,000 g for 5 minutes. For estimation of the maximal increase in LDH activity caused by lysis of the platelets, digitonin (50 $\mu\text{mol/L}$) was added to separate samples of PRP immediately after addition of NaCl or NaF. These samples were further processed as detailed previously.

RESULTS

Effect of NaF on platelet aggregation and ATP release. NaF caused irreversible aggregation of platelets and secretion of platelet granule contents as measured by ATP release in a strictly dose-related manner (Fig 1). The peak aggregation response was observed at NaF concentrations of ~30 mmol/L. At higher concentrations, the onset of aggregation was more rapid; maximal increase in light transmission was reduced, however, and aggregation was almost entirely inhibited at NaF concentrations >70 mmol/L. Moreover, at these concentrations, platelets became refractory to subsequent stimulation by thrombin, collagen, adenosine 5'-diphosphate (ADP), arachidonic acid, platelet-

activating factor (PAF), and calcium ionophore A23187 added in threshold concentrations 1 minute after addition of NaF (data not shown). Threshold concentrations of these agonists were determined in NaCl-treated control samples. Shape change, reflected by the initial decrease in light transmission, occurred at all concentrations tested. As illustrated in Fig 1B, platelet shape change was delayed and prolonged at low concentrations of NaF whereas it occurred almost instantaneously at high concentrations. Platelet aggregation induced by 30 mmol/L of NaF was not inhibited by apyrase, acetylsalicylic acid, or the PAF antagonist CV-3988,³⁰ in concentrations sufficient to inhibit aggregation by threshold concentrations of ADP, arachidonic acid, and PAF, respectively. In addition, the aggregation response to 30 mmol/L of NaF was not blocked by preincubation of PRP with a combination of high concentrations of apyrase (5 mg/mL), acetylsalicylic acid (0.2 mmol/L), and CV-3988 (20 $\mu\text{mol/L}$) for 3 minutes (data not shown), indicating that the NaF-induced aggregation was independent of ADP release, arachidonic acid metabolism, or endogenously synthesized PAF. The concentration-response curve of NaF-induced ATP release essentially paralleled that of the aggregation response, with the exception that its peak was shifted to 40 mmol/L of NaF and that, at high concentrations of NaF that almost completely inhibited aggregation, there was still a marked release of ATP. ATP release occurred more rapidly with increasing concentrations of NaF.

Time-related and concentration-related effects of NaF on inositol phosphate formation. NaF-induced phospholipid cleavage of platelet membrane phosphoinositides was monitored by measuring the formation of radioactive inositol phosphates in [^3H]inositol-labeled platelets. As demonstrated in time-progress experiments (Fig 2), NaF at low (30 mmol/L) and high (80 mmol/L) concentrations caused a rise in inositol 1-phosphate (IP1) and inositol 1,4-bisphosphate (IP2) levels. The increase in IP2 was detectable after 30 seconds at both concentrations tested and preceded the rise in IP1. The formation of both IP1 and IP2 appeared to be

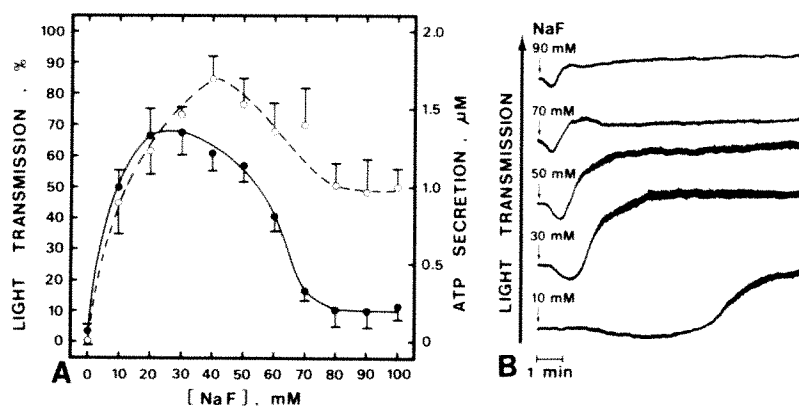


Fig 1. Effect of NaF on platelet aggregation and ATP release. Citrated platelet-rich plasma (PRP) was exposed to increasing concentrations of NaF in the presence of luciferin/luciferase reagent in a Lumi-Aggregometer as described in the Materials and Methods section. Panel A shows the maximal increase in light transmission (●) reflecting platelet aggregation and the maximal plasma concentrations of adenosine triphosphate (ATP) (○) released from platelets measured during an observation period of 10 minutes. The difference in light transmission between PRP and PPP was set equal to 100%. Points represent means $\pm$ SE of four experiments. Panel B shows representative tracings of platelet aggregation induced by different concentrations of NaF.

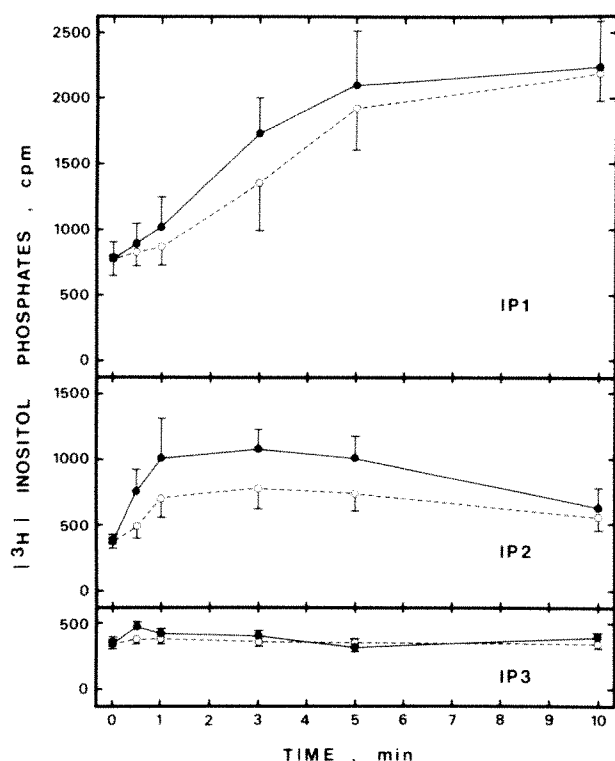


Fig 2. Time course of NaF-induced changes in inositol phosphate formation. [^3H]Inositol-labeled platelets were incubated with either 30 mmol/L of NaF + 70 mmol/L of NaCl ($\circ$) or 80 mmol/L of NaF + 20 mmol/L of NaCl ($\bullet$) for varying periods of time. For determination of zero time values ($\blacksquare$), 100 mmol/L of NaCl was added to the samples and the reaction was stopped immediately. Formation of radioactive inositol phosphates was measured as detailed in the Materials and Methods section. Points represent means $\pm$ SE of five experiments. IP1, inositol 1-phosphate; IP2, inositol 1,4-bisphosphate; IP3, inositol 1,4,5-trisphosphate.

delayed at lower concentrations of NaF. This observation was confirmed by the findings of dose-response experiments in which the concentration-dependent differences in inositol phosphate formation were compensated by prolongation of the incubation time from 5 to 10 minutes (Fig 3). A small increase in inositol 1,4,5-trisphosphate (IP3) was measured within 30 seconds of addition of 80 mmol/L of NaF. No clear-cut changes in IP3 formation were detected when platelets were stimulated with lower concentrations of NaF, however, NaCl in a final concentration of 100 mmol/L did not affect inositol phosphate formation (data not shown).

Effect of NaF on protein phosphorylation. Preliminary time-course experiments (data not shown) revealed that the 40-kd protein was maximally phosphorylated within about 4 minutes of addition of 100 mmol/L of NaF. In dose-response experiments (Fig 4), ^{32}P -labeled platelets were therefore incubated with increasing concentrations of NaF for either 1, 4, or 10 minutes. A minor rise in ^{32}P content of the 40-kd protein after incubation for 1 minute was observed only at NaF concentrations of ≥ 40 mmol/L. At these concentrations, 40-kd protein phosphorylation was maximal after 4 minutes, yielding $\sim 350\%$ to 500% of control values, and a marked dephosphorylation was measured on further

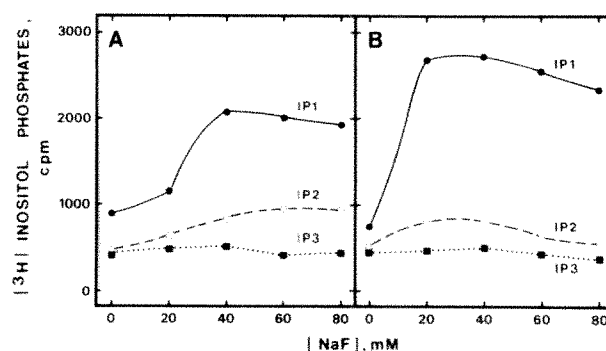


Fig 3. Concentration-related and time-related effects of NaF on inositol phosphate formation. [^3H]Inositol-labeled platelets were incubated with different concentrations of NaF as indicated for either 5 (A) or 10 minutes (B); 100 mmol/L of NaCl was added to controls (0 mmol/L of NaF). IP1 ($\bullet$), IP2 ($\circ$), and IP3 ($\blacksquare$) were determined as described in the Materials and Methods section. A representative experiment is shown. Abbreviations as in legend to Fig 2.

prolongation of the incubation time to 10 minutes. In contrast, at low concentrations of NaF, ^{32}P incorporation into the 40-kd protein continued to rise when the incubation time was extended from 4 to 10 minutes, reaching a maximum of $\sim 450\%$ of control values. These data indicate that the rate rather than the maximal degree of 40-kd protein phosphorylation was concentration-dependent. Only minor changes in ^{32}P incorporation into the 20-kd protein were observed (data not shown), which was in accordance with the relatively small rises in $[\text{Ca}^{2+}]_i$ induced by NaF (see below); 20-kd

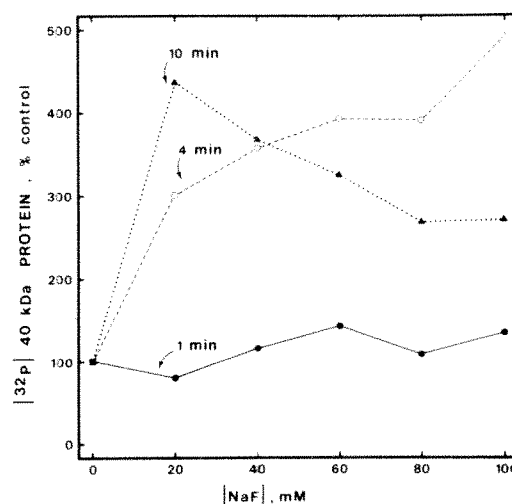


Fig 4. Concentration-related and time-related effects of NaF on 40-kd protein phosphorylation. ^{32}P -labeled platelets were incubated while being stirred with various concentrations of NaF for either 1 minute ($\bullet$), 4 minutes ($\circ$), or 10 minutes ($\blacktriangle$). Isolated platelet proteins were separated by electrophoresis on 12% sodium dodecyl sulphate-polyacrylamide gels, and radioactivity in specific areas of the gels corresponding to the 40-kd protein was determined as described in the Materials and Methods section; 100 mmol/L of NaCl was added to controls (0 mmol/L of NaF). ^{32}P Radioactivity in the 40-kd protein bands in controls was set equal to 100% and ranged between 440 and 850 cpm. The data shown are representative of two experiments.

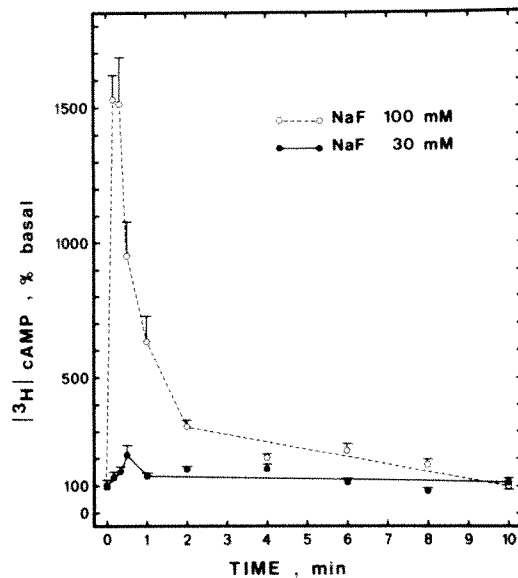


Fig 5. Time-course of NaF-induced changes in platelet cAMP levels. [^3H]Adenine-labeled platelets were incubated with either 30 mmol/L of NaF + 70 mmol/L of NaCl (●) or 100 mmol/L of NaF (○) for various periods of time as indicated, and [^3H]cAMP was determined as detailed in the Materials and Methods section. Basal [^3H]adenine incorporation into cAMP was measured after incubation of platelets with 0.1 vol of 1 mol/L of NaCl for 20 seconds and was set equal to 100%. Values are given as means $\pm$ SE of triplicate incubations. The data shown are representative of four experiments.

protein phosphorylation essentially paralleled cytosolic free Ca^{2+} fluxes.

Time-related and concentration-related effects of NaF on cytosolic free Ca^{2+} and platelet cAMP levels. As illustrated in Fig 5, NaF promoted a dose-dependent transient rise in platelet cAMP levels. Net cAMP formation peaked already within 10 to 20 seconds of addition of 100 mmol/L of NaF, whereas the much smaller maximal increment in platelets when stimulated with 30 mmol/L of NaF was observed after 30 seconds. Thus, it is likely that the results of dose-response experiments in which platelets were incubated with different concentrations of NaF for 20 seconds underestimate to a minor extent the effect of low concentrations of NaF on platelet cAMP levels.

The increase in $[\text{Ca}^{2+}]_i$ observed when platelets were stimulated with NaF was preceded by a lag period which in its length was inversely related to the concentration of NaF used. For evaluation of concentration-related effects of NaF on $[\text{Ca}^{2+}]_i$, peak $[\text{Ca}^{2+}]_i$ was calculated from the maximal Quin 2 fluorescence signal observed during 10 minutes following the addition of NaF.

Figure 6 gives an integrated view on the dose-related effects of NaF on peak $[\text{Ca}^{2+}]_i$ and peak cAMP levels. At low concentrations of NaF (<40 mmol/L), net cAMP formation was only little affected, whereas $[\text{Ca}^{2+}]_i$ increased in a dose-dependent manner to maximally 200% to 250% of basal values. In contrast, at concentrations >40 mmol/L, there was a marked depression of NaF-induced Ca^{2+} mobilization that coincided with a dose-dependent exponential increase in peak cAMP levels. Because [^3H]cAMP formation was deter-

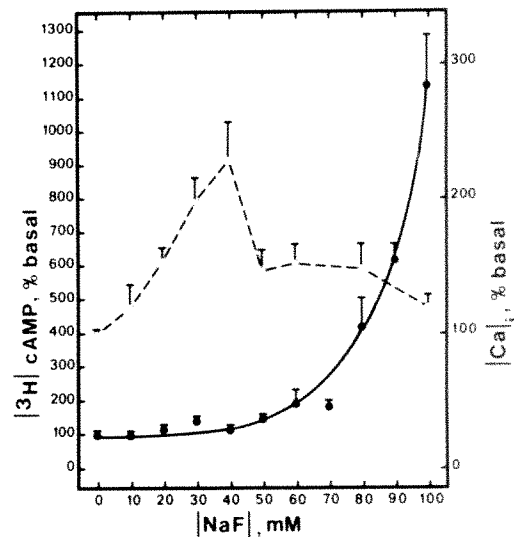


Fig 6. Concentration-dependent effects of NaF on platelet peak cAMP levels (●) and cytosolic free Ca^{2+} concentrations (○). [^3H]Adenine-labeled platelets were incubated with increasing concentrations of NaF for 20 seconds, and [^3H]cAMP was determined. Results are expressed in percentage of basal [^3H]adenine incorporation into cAMP and are given as means $\pm$ SE of triplicate incubations (experimental details are described in the Materials and Methods section). The experiment shown is representative of four. $[\text{Ca}^{2+}]_i$ was measured in gel-filtered platelets suspended in low $[\text{Ca}^{2+}]_i$ -containing buffer using the fluorescent Ca^{2+} probe Quin 2. Results reflect maximal $[\text{Ca}^{2+}]_i$ measured during an observation period of 10 minutes after addition of various concentrations of NaF and are expressed in percentage of basal $[\text{Ca}^{2+}]_i$. Points represent means $\pm$ SE of at least three experiments.

mined in PRP and $[\text{Ca}^{2+}]_i$ was measured in platelets suspended in low $[\text{Ca}^{2+}]_i$ -containing buffer, a direct comparison of these dose-response curves may be somewhat artificial. Nevertheless, the data strongly point to an inverse relationship between NaF-induced cAMP formation and Ca^{2+} mobilization.

NaF-induced inhibition of adenylate cyclase. The preceding data show that NaF-treatment of platelets results in a transient rise in cAMP that is followed by a rapid decline to slightly elevated or basal levels (Fig 5). A similar time course was observed in the presence of PGI_2 (Fig 7). Incubation of platelets with 0.05 $\mu\text{mol/L}$ of PGI_2 resulted in a rapid rise in cAMP levels that reached a maximum after 3 minutes and only gradually decreased during the following 10 minutes. Addition of 30 or 100 mmol/L of NaF to platelets that were preincubated with 0.05 $\mu\text{mol/L}$ of PGI_2 for 3 minutes promoted a marked further dose-dependent increase in platelet cAMP levels that peaked after 10 seconds. At both NaF concentrations tested, however, the temporary enhancement of PGI_2 -induced cAMP formation was followed by a rapid decrease in cAMP to clearly below the levels maintained by stimulation with PGI_2 alone. These data were suggestive of either an NaF-induced activation of a cAMP phosphodiesterase or a delayed inhibitory effect of NaF on adenylate cyclase activity. When platelets were preincubated with 10 mmol/L of MIX, a potent cAMP phosphodiesterase inhibitor,³¹ for 4 minutes prior to exposure to either 100 mmol/L of NaCl or 30 mmol/L of NaF and 70 mmol/L of NaCl for an

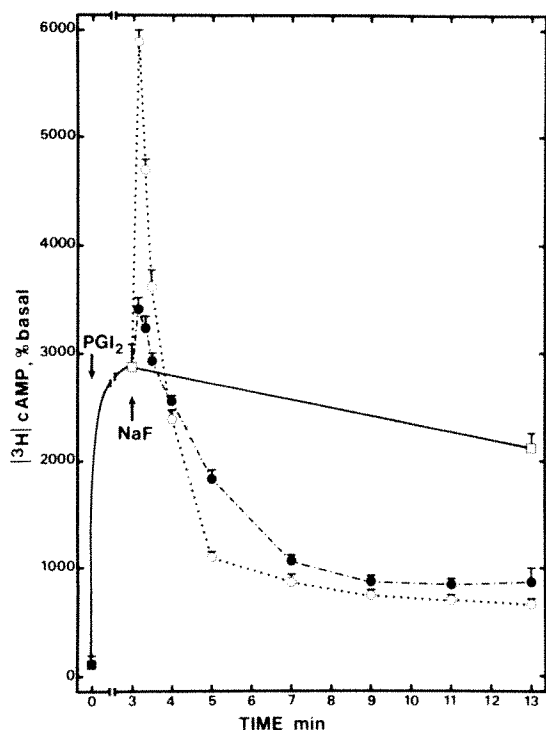


Fig 7. Effect of NaF on prostacyclin (PGI_2)-stimulated cAMP accumulation. [^3H]Adenine-labeled platelets were incubated with 0.05 $\mu\text{mol/L}$ of PGI_2 for 3 minutes prior to addition of either 100 mmol/L of NaCl ($\square$), 30 mmol/L of NaF + 70 mmol/L of NaCl ($\bullet$), or 100 mmol/L of NaF ($\circ$). At the time points indicated, [^3H]cAMP was determined as described in the Materials and Methods section. Results are expressed in percentage of basal [^3H]adenine incorporation into cAMP and are given as means $\pm$ SE of triplicate incubations. The data shown are representative of three experiments.

additional 4.5 minutes, a slow progressive rise in platelet cAMP levels attributable to cAMP phosphodiesterase inhibition was observed (Fig 8). The slightly larger rise measured on addition of NaF was probably due to minor stimulation of adenylate cyclase. When finally 0.05 $\mu\text{mol/L}$ of PGI_2 was added, cAMP levels progressively increased over the next 10 minutes in the NaCl-treated controls. In the NaF-treated samples, the further increase in cAMP achieved by addition of PGI_2 was strikingly smaller, indicating that NaF indeed exerted a delayed inhibitory effect on adenylate cyclase. Our data, however, do not rule out that NaF, in addition, stimulates a low-affinity cAMP phosphodiesterase.

LDH measurements. Incubation of platelets with either 100 mmol/L of NaCl or 100 mmol/L of NaF did not cause a detectable rise in total LDH activity in citrated plasma, indicating that neither treatment caused measurable lysis of platelets.

DISCUSSION

We showed that NaF at optimal concentrations causes irreversible aggregation of platelets and secretion of granule contents as measured by ATP-release. The aggregation induced by NaF cannot be blocked by a combination of high

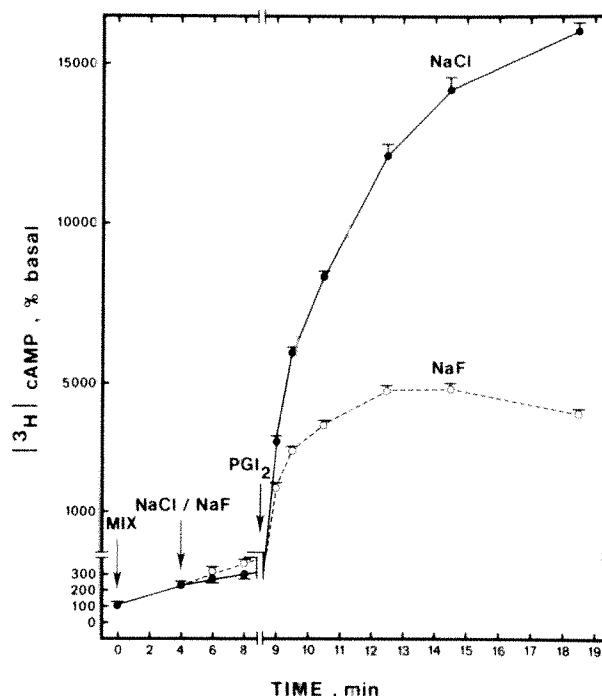


Fig 8. Effect of NaF on prostacyclin (PGI_2)-induced stimulation of adenylate cyclase. [^3H]Adenine-labeled platelets were incubated with 10 mmol/L of 3-isobutyl-1-methyl xanthine (MIX) for 4 minutes prior to addition of either 100 mmol/L of NaCl ($\bullet$) or 30 mmol/L of NaF + 70 mmol/L of NaCl ($\circ$). After an additional 4.5 minutes, adenylate cyclase was stimulated by 0.05 $\mu\text{mol/L}$ of PGI_2 . Samples for determination of [^3H]cAMP (described in the Materials and Methods section) were taken at various time points as indicated. Results are expressed in percentage of basal [^3H]adenine incorporation into cAMP and are given as means $\pm$ SE of triplicate incubations. The data shown are representative of three experiments.

concentrations of specific inhibitors that entirely abolishes aggregation by arachidonic acid, ADP, and PAF, respectively. Thus, it can be assumed that NaF exerts its agonistic effects on platelet function through a mechanism that is independent of the formation of cyclooxygenase products or the release of ADP or PAF.

An early event in agonist-induced platelet activation is the phospholipase C-catalyzed hydrolysis of phosphatidylinositol and its phosphorylated derivatives.^{3,4,32} This results in the generation of intermediate breakdown products that function as intracellular messenger molecules, among them IP₃, which mobilizes Ca^{2+} from intracellular storage sites,^{33,34} and diacylglycerol, the endogenous activator of protein kinase C.^{35,36} Stimulation of platelets by NaF increases the formation of inositol phosphates, indicating the activation of a phosphoinositide-specific phospholipase C. NaF-induced phosphoinositide turnover leads to activation of protein kinase C, as demonstrated by phosphorylation of its major substrate in platelets, a 40-kd protein,^{4,37,38} and a rise in cytosolic free Ca^{2+} , which in turn triggers the phosphorylation of a 20-kd protein previously identified as a myosin light chain.³⁹ In this study, we failed to detect any clear-cut changes in IP₃ formation at NaF concentrations that evoked maximal Ca^{2+} mobilization. Because the rise in IP₃ even on

stimulation of platelets by thrombin is only transient and minor as compared with the increase in IP₂ and IP₁,¹⁹ however, it is possible that the assay used is not sensitive enough to detect a slow rise in IP₃ which, in addition, could be outbalanced by its rapid degradation. Therefore, the most likely explanation for the agonistic effects of NaF on platelet function would still be that NaF through activation of phospholipase C initiates an intracellular signal cascade that finally leads to platelet aggregation and release of granule contents. On the other hand, our data do not entirely exclude the existence of a Ca²⁺-mobilizing agent distinct from IP₃ that is formed or activated on NaF stimulation of platelets.

The time lag that precedes the NaF effects related to platelet activation decreases with increasing concentrations of NaF and may thus reflect the time required for NaF to reach its site of action either within the platelet membrane or possibly within the platelet cytosol. Our data herein are consistent with previous studies reporting a delayed mobilization of cytosolic free Ca²⁺ in platelets,¹⁵ neutrophils,⁴⁰ and hepatocytes⁴¹ exposed to NaF.

When the concentration of NaF is raised to >40 mmol/L, a dose-dependent reduction in the maximal amplitude of platelet aggregation is observed, and pretreatment of platelets with 70 to 100 mmol/L of NaF even prevents aggregation in response to stimulation by various other agonists. At these concentrations, NaF induces a dose-dependent transient rise in platelet cAMP, which probably accounts for the concomitant reduction in Ca²⁺ mobilization.⁴²⁻⁴⁴ Inositol phosphate formation and 40-kD protein phosphorylation are not affected by the cAMP transients, suggesting that neither phospholipase C nor protein kinase C action is inhibited by a short-lived increase in cAMP levels. We thus conclude that the observed inhibition of platelet function at high concentrations of NaF is due to the increase in cAMP, which counteracts the mobilization of cytosolic free Ca²⁺ and mainly thereby inhibits aggregation and release reaction. Alternatively, NaF at high concentrations might interfere with the formation or activation of a Ca²⁺-mobilizing messenger distinct from IP₃ or directly stimulate a Ca²⁺ pumping activity, thus reducing the cytosolic free Ca²⁺ concentration.

Platelet shape change is not and ATP release is only partially inhibited even at the highest concentrations of NaF tested, which would confirm the earlier observations that shape change and release reaction can occur with virtually no detectable rise in cytosolic free Ca²⁺ as measured by the fluorescent Ca²⁺ indicator Quin 2.^{26,45}

The rapid reversal of the NaF-stimulated increase in platelet cAMP levels raises the hypothesis that NaF may, in addition, either activate a cAMP phosphodiesterase or exert a delayed inhibitory effect on adenylate cyclase. The latter possibility is confirmed by the observation that NaF-pretreatment of platelets partially inhibits PGI₂-induced cAMP accumulation in the presence of MIX; this experiment, however, does not preclude an additional effect of NaF on a cAMP phosphodiesterase.

Because in platelet membrane preparations NaF has been shown to activate the stimulatory (N_s) as well as the inhibitory (N_i) guanine nucleotide-binding protein involved in the regulation of adenylate cyclase activity,^{12,13} it is possible that the dual effect of NaF on adenylate cyclase in intact platelets mirrors the concentration-dependent, concomitant, or successive activation of both N_s and N_i. In extension of this hypothesis, we further suggest that a NaF-sensitive N protein may also play a role in the activation of phospholipase C. We observed activation of phospholipase C by NaF independent of a rise in cytosolic free Ca²⁺ and despite high cAMP transients. In granulocyte membranes, an N protein is required for the stimulation of phosphoinositide-specific phospholipase C at resting cytosolic free Ca²⁺ concentrations.⁴⁶

In conclusion, we demonstrated that NaF, as a result of simultaneous modulation of phospholipase C and adenylate cyclase activity, has a biphasic, concentration-dependent effect on the function of intact human platelets. The action of NaF may be linked to its known capacity to activate N proteins.

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Factor XIIIa Formation Promoted by Complexing of α -Thrombin, Fibrin, and Plasma Factor XIII

By Charles S. Greenberg, Komandoor E. Achyuthan, and John W. Fenton II

Fibrin polymers (des A,B fibrinogen) reduced the concentration of α -thrombin required for 50% activation of plasma factor XIII (a_2b_2 tetramer) by approximately 100-fold. In the presence of fibrin, the amount of γ -thrombin required for activation was not affected. Catalytically inactive *i*-Pr₂P- and D-Phe-Pro-Arg-CH₂- α -thrombin were found to inhibit over 95% of the activation by α -thrombin in the presence of fibrin. Unlike plasma factor XIII, the concentration of α -thrombin required for 50% activation of platelet factor XIII (a_2 dimer) was lower, and the activation was not enhanced by fibrin. However, when the a_2 platelet factor XIII was incubated with purified b-chains, the α - and γ -thrombin concentrations required for activation increased tenfold

and reached levels similar to those required for activation of the plasma factor XIII. When fibrin was present, the α -thrombin concentrations needed for activation of the a_2b_2 complexes were reduced, and the presence of fibrin had no effect on γ -thrombin cleavage of the a_2b_2 complexes. Therefore, the b-chains must inhibit a-chain cleavage by α -thrombin in the absence of fibrin. These results imply that the formation of a cocomplex involving α -thrombin, fibrin, and plasma factor XIII causes some conformational change in plasma factor XIII such that the b-chains no longer inhibit cleavage of the a-chains.

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PLASMA FACTOR XIII (a_2b_2) is composed of two "a" and two "b" polypeptide chains that form a tetramer in circulating blood. Upon cleavage of factor XIII, the activation peptides are released and form an inactive intermediate, factor XIII' (a_2b_2).¹ In the presence of Ca²⁺ and fibrin(ogen), factor XIII' undergoes a conformational change that results in the dissociation of b-chains and the formation of factor XIIIa.² In this process, the presence of fibrin(ogen) reduces the Ca²⁺ concentration required for b-chain dissociation³ and also enhances thrombin-catalyzed release of the activation peptides from the a-chains of purified plasma factor XIII.⁴

In our studies examining the factors affecting the activation process, we found that α -thrombin would cause a 43-fold more rapid release of fibrinopeptide A from fibrinogen than activation of plasma factor XIII. Previous studies had found that a temporal relationship existed between fibrin polymerization and factor XIII activation and that inhibition of fibrin polymerization delayed the activation event without inhibiting fibrinopeptide A release.⁵ In addition, the polymerization of des-A-fibrinogen and des-A,B-fibrinogen accelerated the rate of thrombin cleavage of plasma factor XIII in afibrinogenemic plasma,⁶ and inhibition of fibrin polymerization by Gly-Pro-Arg-Pro prevented fibrin polymerization and significantly reduced the rate of plasma factor XIII cleavage by thrombin.^{5,6} Furthermore, the plasma factor XIII was found to bind specifically to polymerized fibrinogen and fibrin fibers, and the binding was not affected by other plasma proteins or divalent cations.⁷

Unlike plasma factor XIII, platelet factor XIII is composed of two a-chains alone yet binds like its plasma counterpart to fibrin(ogen), which suggests that the a_2 -subunit may mediate binding.^{7,8} Since fibrin(ogen) has been found to selectively bind α -thrombin,⁹⁻¹³ we have proposed that the formation of a cocomplex involving thrombin, fibrin, and plasma factor XIII promotes the formation of factor XIIIa.⁶ Unlike α -thrombin, γ -thrombin lacks clotting activity and binds very weakly to fibrin(ogen)^{10,11} yet retains its catalytic activity and cleaves factor XIII.¹⁴ When the catalytic serine and histidine residues are covalently conjugated with *i*-Pr₂P-F or D-Phe-Pro-Arg-CH₂-Cl, α -thrombin becomes irreversibly inactivated.^{15,16} These α -thrombin conjugates cannot cleave plasma factor XIII or clot fibrinogen solutions, yet they can bind to fibrin(ogen) and compete for α -

thrombin binding sites.¹⁰⁻¹² In the present study, we used modified thrombin to explore the mechanisms of fibrin-mediated enhancement of plasma factor XIII activation. The binding of both α -thrombin and plasma factor XIII to fibrin was found to be required for the rapid activation of plasma factor XIII.

MATERIALS AND METHODS

Factor XIII-deficient plasma was purchased from George King Biomedical (Overland Park, KS) and adsorbed with 15 mg/mL of bentonite (Sigma Chemical Co, St Louis) for 30 minutes at 22 °C.⁶ Human plasma and platelet factor XIII were purified from fresh, frozen plasma and stored at -70 °C.^{17,18} Fibrinogen and des-A,B-fibrinogen were prepared as previously described,⁶ and D-Phe-Pro-Arg-CH₂-Cl, *i*-Pr₂P-F, anti-factor XIII a-chain and anti-factor XIII b-chain antibodies were purchased from Calbiochem-Behring Corp (La Jolla, CA).

Thrombin preparations. Human α -thrombin was purified from fraction III paste and stored frozen at -70 °C in 0.75 mol/L NaCl, pH 6.0. This preparation contained a final protein concentration of 69.7 μ mol/L, an active site titration of 68.2 μ mol/L, and fibrinogen clotting activity of 3,100 US National Institutes of Health (NIH) U/mg.¹⁹ γ -thrombin was prepared by passing purified α -thrombin through a trypsin-agarose column²⁰ and stored as noted before (52 μ mol/L protein, 24.8 μ mol/L active site titration, and 0.36 NIH U/mg activity). Inactivated *i*-Pr₂P- α -thrombin was prepared by repeated additions of the fluoride reagent to α -thrombin until the clotting activity declined to <2%. This was followed by extensive

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dialysis against 0.75 mol/L NaCl at 4 °C,⁹ and the enzyme was stored frozen (52 μ mol/L protein, <0.5 μ mol/L active site titration, and 0.37 NIH U/mg activity). Affinity-labeled D-Phe-Pro-Arg-CH₂- α -thrombin was made by incubating equimolar amounts of the chloromethyl ketone reagent and α -thrombin for one hour followed by dialysis to remove unreacted reagent. Protein concentrations were calculated from absorbance at 280 nm by using an extinction coefficient of 1.83 mg/mL/cm and a molecular weight of 36,500 for α -thrombin.¹⁹ All concentrations of reagents used in the study were based upon the absolute protein concentrations rather than the active site concentrations.

Measurement of α - and γ -thrombin cleavage of plasma and platelet factor XIII in the presence and absence of des-A,B-fibrinogen. α - or γ -thrombin at 0.01 to 1,000 nmol/L was incubated with 6 nmol/L platelet factor XIII or 9 nmol/L plasma factor XIII for five minutes at 37 °C in the presence or absence of 620 nmol/L des-A,B-fibrinogen in Tris-buffered saline (0.13 mol/L NaCl at pH 7.4 and 0.02 mol/L Tris-HCl (TBS), and 5 mmol/L CaCl₂). Thrombin was inhibited by a 100-fold molar excess of D-Phe-Pro-Arg-CH₂-Cl, and factor XIIIa was assayed after stabilization in a buffer containing glycerol at 4 °C.²¹ Preliminary experiments demonstrated that the presence of D-Phe-Pro-Arg-CH₂-Cl did not interfere with the factor XIIIa assay. Less than 5% of the factor XIII preparation expressed activity in the absence of α -thrombin, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis demonstrated over 87% cleavage of the a-chains by thrombin in the factor XIII preparations.⁵

Factor XIIIa assay. Plasma and platelet factor XIIIa formation was quantitated using the [³H]-putrescine incorporation assay.²¹ Factor XIIIa activity was expressed as nanomoles of putrescine incorporated into casein in 60 minutes at 37 °C, where this activity correlated with thrombin cleavage of the a-chains.⁵ Factor XIIIa activity was expressed as the percentage of total factor XIIIa determined after incubating samples with 5 mmol/L CaCl₂ and 190 nmol/L α -thrombin at 37 °C for 15 minutes.

Effect of *i*-Pr₂P- and D-Phe-Pro-Arg-CH₂-thrombin on α -thrombin cleavage of plasma factor XIII in the presence or absence of fibrinogen. Concentrations of either *i*-Pr₂P-thrombin or D-Phe-Pro-Arg-CH₂-thrombin ranging from 9 to 90 nmol/L were incubated at 22 °C for five minutes in TBS containing 9 nmol/L plasma factor XIII and the presence or absence of 620 nmol/L fibrinogen. These reaction mixtures were subsequently incubated for five minutes at 37 °C with 9 nmol/L α -thrombin followed by the addition of 1 mmol/L D-Phe-Pro-Arg-CH₂-Cl. Factor XIIIa activity was measured by the [³H]-putrescine incorporation assay. Samples of the reaction mixtures that contained fibrinogen were further ultrafiltered through an Amicon ultrafiltration cell using an X M 100A membrane (Amicon Corp, Danvers, MA) to determine whether the effect of the modified thrombins was reversible.

Effect of plasma factor XIII b-chains on α -thrombin cleavage of factor XIII a-chains. Plasma containing only factor XIII b-chains was prepared by bentonite adsorption of factor XIII-deficient plasma. The adsorbed plasma was electrophoresed through agarose gels containing either anti-a-chain or anti-b-chain antisera to demonstrate the absence of a-chains and the presence of b-chains.²² Equal volumes of 0.24 μ mol/L platelet factor XIII (a₂) and bentonite-adsorbed factor XIII-deficient plasma were incubated for 15 minutes at 22 °C. Following incubation, the samples were diluted to 20% vol/vol with TBS and incubated for five minutes at 37 °C with 0.01 nmol/L to 10 nmol/L α -thrombin and in the presence or absence of 0.62 μ mol/L des A,B fibrinogen. In another set of reaction mixtures, purified factor XIII b-chains²³ at 3.6 nmol/L were incubated with 2.4 nmol/L platelet factor XIII a-chains in TBS for 15 minutes at 22 °C. To these, either 0.01 to 10 nmol/L of α -thrombin or 0.1 to 100 nmol/L γ -thrombin was added, and the

samples were incubated for 15 minutes at 37 °C in the presence or absence of 620 nmol/L des A,B fibrinogen. The reactions were stopped by the addition of D-Phe-Pro-Arg-CH₂-Cl to 1 μ mol/L, and the level of factor XIIIa formation was determined by the [³H]-putrescine incorporation assay.

Formation of a₂b₂ complex (plasma factor XIII) by binding of b-chains to a-chains. Platelet factor XIII was radioiodinated with Na¹²⁵I using lactoperoxidase to a level of 5,620 cpm/ng.⁸ The radiolabeled a-chains were incubated at 6 nmol/L with 80 nmol/L purified b-chains for 15 minutes at 22 °C. Complexes formed in the reaction mixture as well as radiolabeled plasma factor XIII (2,680 cpm/ng) were electrophoresed through discontinuous PAGE.² The gels were stained and dried, and autoradiography was performed to identify the location of radiolabeled factor XIII a-chains bound to purified b-chains. Autoradiograms were scanned using a GS-300 scanning densitometer (Hoefer, San Francisco) to quantitate the amount of ¹²⁵I-labeled a-chains that had complexed with b-chains.

Measurement of fibrin polymerization. Purified fibrinogen at 2 mg/mL was incubated with 10 nmol/L of α -thrombin in TBS containing 5 mmol/L CaCl₂. The level of fibrin polymerization was measured as previously described,²¹ and the rate of polymerization was calculated as a function of the maximum slope of the polymerization curve.

RESULTS

Fibrin polymers generated from des A,B fibrinogen reduced the level of α -thrombin required for 50% activation of plasma factor XIII by approximately 100-fold. In contrast, the level of γ -thrombin required for 50% activation had slightly increased in the presence of fibrin (Fig 1). On a functional enzyme basis, the α -thrombin and γ -thrombin preparations were 97.8% active (μ mol/L active site/ μ mol/L protein $\times$ 100) and 47.7% active, respectively. Therefore, α -thrombin exhibited an approximate 15-fold increase over γ -thrombin in its ability to activate the plasma factor XIII in the absence of fibrin and an increase greater than two orders of magnitude over γ -thrombin in the presence of fibrin. In control experiments, 1000 nmol/L γ -thrombin could not

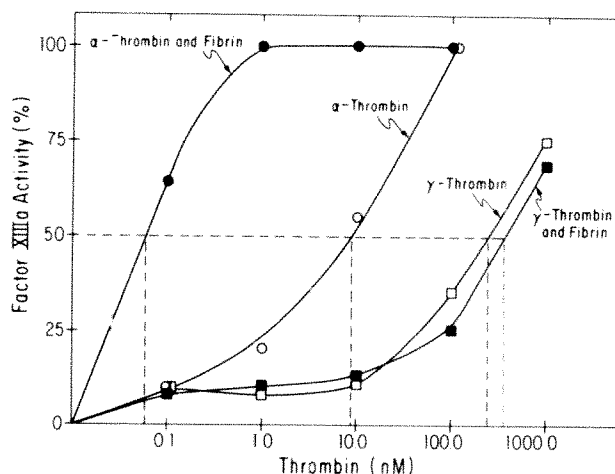


Fig 1. Plasma factor XIII activation by α - or γ -thrombin in the presence or absence of fibrin polymers. Factor XIII was activated and assayed as described in the text; it was incubated with α -thrombin (○—○) or γ -thrombin (□—□) alone (open symbols) or plus des A,B fibrinogen (solid symbols).

inhibit activation of the plasma factor by 1.0 nmol/L α -thrombin in either the presence or absence of fibrin.

Since the fibrin used in the aforementioned experiments was generated by diluting ten- to 20-fold des A,B fibrinogen in 1 mol/L NaBr, the high salt concentrations could potentially interfere with thrombin binding to fibrin(ogen). To address this issue, experiments were conducted with fibrin generated in situ from fibrinogen reacted with α -thrombin. We found that a tenfold molar excess of inactivated *i*-Pr₂P- or D-Phe-Pro-Arg-CH₂- α -thrombin inhibited over 95% of the activation of plasma factor XIII by α -thrombin in the presence of fibrin(ogen) (Fig 2), but neither appreciably inhibited factor XIII activation in the absence of fibrin(ogen).

The highest concentrations of catalytically inactivated thrombins did not interfere with fibrin formation by α -thrombin in the fibrinogen-containing solutions. However, when these solutions were subsequently ultrafiltered through membranes with a 100,000-dalton cutoff, they regained their ability to promote α -thrombin activation of factor XIII. Dialyzing the modified-thrombin solutions did not interfere with their ability to competitively inhibit α -thrombin cleavage of the plasma factor XIII with fibrin. In addition, when 1.8 μ mol/L α -thrombin was added to 9 nmol/L plasma factor XIII with 90 nmol/L *i*-Pr₂P- α -thrombin, additional factor XIIIa was formed.

Unlike the plasma factor, platelet factor XIII required approximately tenfold less α -thrombin for 50% activation, and the activity was not enhanced by the presence of fibrin (Fig 3). γ -Thrombin was at least an order of magnitude less active relative to α -thrombin and was essentially unaffected by the presence of fibrin. However, when platelet factor XIII was incubated with either afibrinogenemic plasma containing b-chains or isolated b-chains from plasma factor XIII, the α -thrombin concentration required to activate the a_2b_2 complexes increased by approximately tenfold in the absence

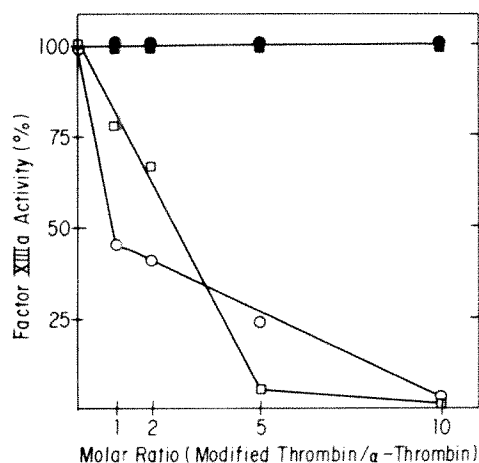


Fig 2. Inhibition of α -thrombin activation of plasma factor XIII with catalytically inactivated thrombins in the presence of fibrin. Des A,B fibrinogen and plasma factor XIII were incubated with increasing concentrations of either *i*-Pr₂P- (○—○) or D-Phe-Pro-Arg-CH₂- α -thrombin (□—□) for 15 minutes prior to assaying factor XIIIa activity, as described in the text. Similar reactions were performed in the absence of fibrin (closed symbols).

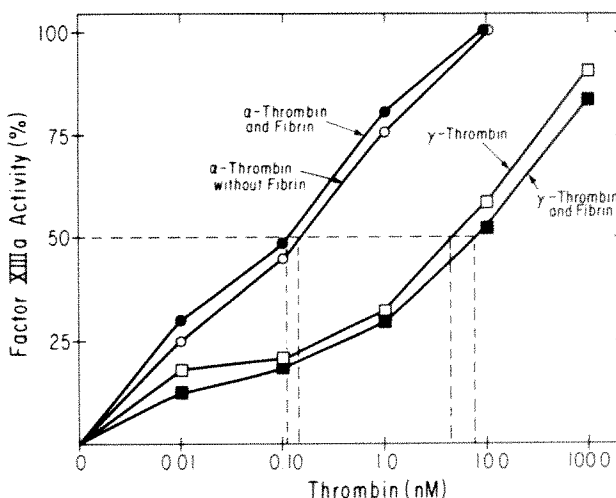


Fig 3. Platelet factor XIII activation by α - or γ -thrombin in the presence or absence of fibrin polymers. See Fig 1: α -thrombin alone (○—○) or α -thrombin plus des A,B fibrinogen (●—●); γ -thrombin alone (□—□) or γ -thrombin plus des A,B fibrinogen (■—■).

of fibrin and was decreased by approximately tenfold in the presence of fibrin (Fig 4). The presence of b-chains inhibited γ -thrombin cleavage of platelet factor XIII (Fig 5), and the presence of fibrin was not found to enhance γ -thrombin cleavage of the a_2b_2 complexes (data not shown).

In additional experiments, platelet factor XIII a-chains were radiolabeled with ¹²⁵I and incubated with isolated factor XIII b-chains. When electrophoresed according to Lorand et al.,² 86% the a-chains migrated as the a_2b_2 complex, which comigrated with plasma factor XIII (data not shown).

DISCUSSION

Fibrin generated from the polymerization of des A,B fibrinogen clearly amplified the α -thrombin activation of plasma factor XIII by approximately 100-fold. This form of the human enzyme exhibits high fibrinogen-clotting activity as well as all other thrombin-associated characteristics.^{19,20} The autoproteolytic or tryptic degradative form of thrombin, γ -thrombin, lacks clotting activity but retains its substrate-binding and certain other enzymic traits.²⁰ Although this form has been shown to activate plasma factor XIII,¹⁴ such activation was not found to be enhanced by the presence of fibrin. This suggests that a fibrin(ogen) recognition site necessary for clotting must be involved in the enhancement phenomenon.

Like α -thrombin, catalytically inactivated forms bind tightly to nonpolymerized fibrin immobilized in agarose and are eluted by similar salt concentrations. Both of the modified forms compete for α -thrombin binding sites and were found to inhibit 95% of the activation of plasma factor XIII by the native enzyme. In contrast, the binding of γ -thrombin to nonpolymerized fibrin is very weak¹¹ and was not found to compete with α -thrombin in the activation of plasma factor XIII. Since the *i*-Pr₂P-group is an obstructive moiety conjugated to the catalytic serine residue and the D-Phe-Pro-Arg-CH₂-group fills the fibrinopeptide groove adjacent to

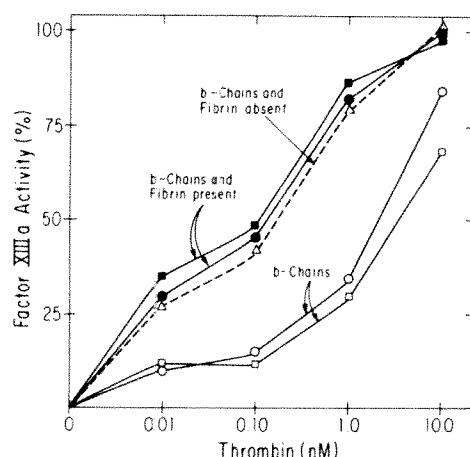


Fig 4. Platelet factor XIII incubated with the plasma factor XIII b-chains and activated with α -thrombin in the presence or absence of fibrin. Platelet factor XIII activated by α -thrombin in the absence of fibrin or b-chain ($\triangle$ — $\triangle$). In addition, plasma containing b-chains with (●—●) or without (○—○) fibrin polymers was incubated with platelet factor XIII and activated with α -thrombin. Isolated b-chains were also incubated with platelet factor XIII and activated by thrombin with (■—■) or without (□—□) fibrin polymers.

the catalytic site,^{15,16} an exosite independent of the catalytic site and adjacent regions must be required for α -thrombin complexing with fibrin(ogen).

The presence of fibrin was not found to enhance the activation of platelet factor XIII by α -thrombin. However, when the platelet factor, which has an a_2 -chain structure, was incubated with b-chains from the plasma factor XIII, an a_2b_2 complex was formed. The activation of this hybrid complex by α -thrombin was enhanced by approximately 20-fold in the presence of fibrin. Although the isolated b-chains cannot complex with fibrin(ogen) alone, yet the a_2 dimers can,^{7,8} the in vitro complexing of a-chains with b-chains clearly inhibited α - and γ -thrombin cleavage of the a-chain when fibrin was absent. Thus, our data strongly suggest that activation of plasma factor XIII proceeds via the formation of cocomplex involving α -thrombin, the a_2b_2 complex, and fibrin.

Earlier investigations suggested that plasma factor XIII b-chains inhibit α -thrombin cleavage of the a-chains.²³ Our data support these findings since approximately tenfold more α -thrombin or γ -thrombin was required to activate plasma factor XIII or the hybrid (a_2b_2) complexes relative to platelet factor XIII (a_2). Our data further demonstrate a previously unrecognized change in the interaction between the a- and b-chains during the clotting event. We have found that the b-chains inhibit cleavage of the a-chains by α - and γ -thrombins in the absence of fibrin. However, in the presence of fibrin, the b-chains no longer inhibit α -thrombin cleavage

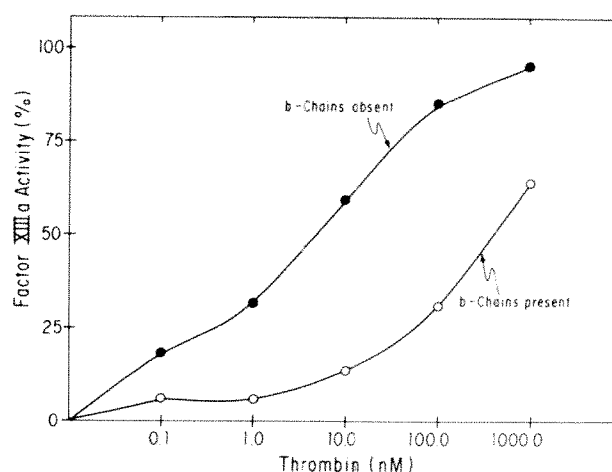


Fig 5. Effect of b-chains on Platelet factor XIII activation by γ -thrombin. Platelet factor XIII was activated by γ -thrombin in the presence (○—○) or absence (●—●) of b-chains. Platelet factor XIII was activated and assayed as described in the text.

of the a-chains. Since the γ -thrombin concentration required to cleave the a-chains was not affected by the presence of fibrin, the binding of thrombin to fibrin must be required for thrombin activation of plasma factor XIII. We propose that the b-chains bind to the a-chains of the a_2b_2 complex, which protects them from thrombin binding in circulating blood. However, in the presence of fibrin, a binding of the a_2b_2 complex to the fibrin polymer leads to a conformational change in the a- and b-chain geometry, which promotes formation of the activation complex.

Preliminary experiments have indicated that non-cross-linked fibrin protofibrils promote α -thrombin activation of plasma factor XIII,²⁴ whereas other data show that soluble, cross-linked fibrin does not.²⁵ Thus, the activation complex appears to occur transiently during fibrin polymerization. This is also true in the active entrapment process whereby α -thrombin becomes incorporated into fibrin clots.¹⁰

The mechanism by which fibrin modifies the interaction between a- and b-chains to promote thrombin cleavage does not appear to involve a requisite dissociation of the b-chains from the complex since des A,B fibrinogen promotes thrombin cleavage of plasma factor XIII in the presence of EDTA under conditions whereby the b-chains do not dissociate from the a-chains.⁶ The b-chain may actively promote a conformational change in the a-chain, or the a-chains may undergo a conformational change that rearranges the position of the b-chains within the complex.

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Durability of Responses to Interferon Alfa-2b in Advanced Hairy Cell Leukemia

By Mark J. Ratain, Harvey M. Golomb, Ronald G. Bardawil, James W. Vardiman, Carol A. Westbrook, Lynne S. Kaminer, Barry C. Lembersky, Mitchell A. Bitter, and Karen Daly

Previous studies have demonstrated that significant hematologic improvement occurs in the majority of patients with hairy cell leukemia (HCL) treated with partially purified or recombinant interferon (IFN). Fifty-three patients received IFN alfa-2b for at least 3 months in a dose of 2×10^6 U/m² subcutaneously thrice weekly. Of the 49 patients evaluable for response (at least 6 months of IFN therapy), there were ten complete responses and 29 partial responses for a total response rate of 80%. The peripheral blood counts and bone marrow continued to improve over the course of a full year of therapy. IFN was well tolerated, with no patients discontinuing therapy because of toxicity. Transient myelosuppression occurred in most patients during the first 1 to 2 months of therapy, occasionally precipitating a trans-

fusion requirement. After IFN treatment was discontinued, there was a marked decrease in normal marrow elements and a relative increase in marrow hairy cells. This was associated with a transient increase in normal elements in the peripheral blood. Only one of 24 patients followed after receiving IFN for a median of 8.5 months (range, 3 to 16 months) has required further therapy. We conclude that low-dose IFN alfa-2b is highly effective in advanced HCL; responding patients should be treated for at least 1 year. The decision to initiate a second course of IFN therapy should be based primarily on peripheral blood counts and the clinical status of the patient rather than on the bone marrow.

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HAIRY CELL LEUKEMIA (HCL) was first described by Bouroncle et al in 1958.¹ Splenectomy has long been considered the initial treatment of choice,² but patients with advanced disease postsplenectomy have historically been difficult to treat because of persistent neutropenia following even low-dose chemotherapy.³

Quesada et al⁴ first demonstrated that a partially purified interferon product was highly effective in advanced HCL, and Ratain et al⁵ showed that interferon itself was the active molecule using the recombinant drug. These initial results with recombinant interferon have subsequently been confirmed in a multiinstitution study of 64 patients,⁶ and the results of several large series have recently been reviewed.⁷ The objectives of this analysis from a single institution are to extend and update our experience with interferon alfa-2b (IFN) in advanced HCL and to determine the duration of response to IFN by careful assessment of peripheral blood and bone marrow changes following discontinuation of therapy.

MATERIALS AND METHODS

Patient eligibility. Requirements for inclusion in the study were as follows: (1) pathologically confirmed diagnosis of HCL on the basis of a recent (within 1 month) bone core biopsy; (2) significant

anemia (hemoglobin, <12 g/dL), thrombocytopenia (platelets, <100,000 cells/ μ L), neutropenia (neutrophils, <1,000 cells/ μ L), and/or leukemia (white blood count, >10,000 cells/ μ L with more than 50% hairy cells); (3) prior splenectomy, unless refused by the patient; (4) normal hepatic and renal function; (5) past the nadir of toxicity of any previous therapy (chemotherapy and/or radiotherapy), preferably no treatment in the previous 4 weeks; and (6) informed consent in accordance with procedures defined by the Department of Health and Human Service.

Study design. IFN (Intron A, Schering Corp, Kenilworth, NJ) was supplied as lyophilized powder (10×10^6 and 50×10^6 U/vial) and reconstituted with sterile water. The initial dose of IFN was 2×10^6 U/m² self-administered subcutaneously three times weekly. Dose escalation up to 10×10^6 U/m² was allowed for nonresponders but used in only two patients. Five of the first six patients were initially treated with 10×10^6 U/m², but it was deescalated when the lower dose was shown to be active. Patients were admitted to the hospital for the first two doses to facilitate teaching of IFN reconstitution and administration and to observe for unexpected toxicity.

Patients were seen monthly at the University of Chicago. Treatment was continued for 12 months in responding patients. Patients completing 12 months of IFN therapy after January 1985 were entered in a randomized study comparing an additional 6 months of IFN therapy to observation. Patients were stratified by response (at the time of randomization), as defined in the next section.

While receiving IFN, a complete blood count (and differential) was obtained weekly until patients showed objective evidence of response, then it was done monthly. A chemistry panel was obtained monthly. Bone marrow biopsy specimens were obtained at least every 3 months. Since the neutrophil alkaline phosphatase score (NAP) has previously been shown to be elevated in HCL,⁸ we also determined the NAP every 3 months. After discontinuation of IFN therapy, a complete blood count was obtained monthly. A chemistry panel, NAP, and bone marrow biopsy specimen were obtained every 3 months for the first year of follow-up and then every 6 months.

Response criteria. Patients were considered eligible for response after 6 months of therapy. Although responses occur within 1 to 2 months of starting IFN treatment, prior studies have demonstrated that the absolute neutrophil count (ANC) and hemoglobin level may improve continuously over the first 6 months of therapy.⁶ The response criteria used in this analysis are similar to those reported previously.⁴⁻⁷

A complete response (CR) was defined as (a) normalization of peripheral blood counts (hemoglobin, >12 g/dL; platelets, >100,000 cells/ μ L; and ANC, >1,500 cells/ μ L) and (2) less than 5% hairy cells in the bone marrow biopsy specimen. (Patients with

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focal clumping of hairy cells in the bone marrow were not considered to have a CR).

A partial response (PR) designated normalization of peripheral blood counts but not meeting the pathological criteria for a CR.

A minor response (MR) was defined as normalization of at least one peripheral blood count initially abnormal but not meeting the criteria for a PR. Leukemic patients with only a decrease in WBC to the normal range were also considered to have an MR.

No response (NR) met none of the aforementioned criteria.

Bone marrow studies. All bone marrow biopsy specimens were reviewed initially by a hematopathologist (J.W.V. or M.A.B.). The bone marrow biopsy specimens on all patients not receiving IFN for at least 3 months were rereviewed by two of the authors (R.G.B. and J.W.V.) and analyzed as previously described.⁹ This included differential counts of 2,000 to 5,000 cells on the histological sections.

The volume of each marrow subpopulation was quantitated based on the estimated cellularity and the differential as follows: volume of subpopulation = percent cellularity $\times$ percentage of subpopulation/10,000. This analysis is similar to the stereological analysis of Flandrin et al¹⁰ and our prior use of the hairy cell index (HCI)¹¹: percent cellularity $\times$ percentage of hairy cells/10,000. We have also quantitated the non-hairy cell index (NHCI) at various time points; it was defined as (the percent cellularity/100) - HCI. Occasional bone marrows were severely hypocellular (<10% cellularity), especially after IFN therapy was stopped. Frequently the peripheral blood counts obtained simultaneously suggested that these biopsy specimens were not considered representative of the patient's bone marrow, and thus nine of 56 marrows (19%) obtained after IFN therapy was stopped were excluded from further analysis.

Statistical analysis. Paired parametric (Student's *t*) and nonparametric (Wilcoxon) tests were used to analyze the changes in peripheral blood counts, NAP, and bone marrow subpopulations during and after IFN therapy. Patients with RBC or platelet transfusion requirements were considered to have a hemoglobin or platelet count of zero for the purpose of this analysis. In general, the Wilcoxon test was used to avoid assuming a normal distribution of data. However, the paired *t* test was used to assess the changes in peripheral blood counts after completion of IFN therapy because of the greater power of this test.

RESULTS

Patient characteristics. We have entered 59 patients in our phase II trial from October 12, 1983, to March 15, 1986. Thirty-three of these patients have been reported previously, with follow-up through February 1985.^{5,6,12} Fifty-three of the 59 patients have completed at least 3 months of therapy (as of March, 15, 1986) and are the subject of this paper (Table 1). Four of the remaining six patients have been recently entered in the study. One patient with refractory thrombocytopenia died of an intracerebral hemorrhage during the first week of therapy,⁵ and one patient with advanced HCL and severe emphysema (requiring continuous oxygen) died of pneumonia during the first month of therapy.

As noted in Table 1, four patients did not have a prior splenectomy. One patient refused a splenectomy, consistent with the stated eligibility criteria. In the other three non-splenectomized patients, the decision to use IFN was made on clinical grounds because all three patients had small spleens and a high percentage of hairy cells in the marrow and would not be expected to have a durable response to splenectomy.¹¹

All but one patient had one or more cytopenias or were in

Table 1. Characteristics of 53 Patients Treated With ≥ 3 Months of IFN

Characteristic	Value
Sex	
Male	47
Female	6
Age (yr)	
Range	33-78
Median	53
Previous splenectomy	49/53 (92%)
Time since splenectomy (mo)	
Range	1-199
Median	24
Previous chemotherapy	25/53 (47%)

the leukemic phase of the disease. Forty patients (76%) were anemic (hemoglobin, <12 g/dL), and 19 patients (36%) were receiving RBC transfusions. Twenty-seven patients (51%) were thrombocytopenic (platelets, <100,000 cells/ μ L), and 12 patients (23%) required platelet transfusions. Most patients (70%) were neutropenic, and three patients had less than 100 neutrophils/ μ L prior to IFN therapy. Fourteen patients (26%) were in the leukemic phase of the disease. Five of our patients also had symptomatic bony lesions, including one patient with normal blood counts.

Responses. Of the 53 patients described earlier, 50 patients have completed at least 6 months of therapy. (The other three patients are still receiving IFN). The patient with normal blood counts at study entry, but symptomatic bony lesions, is not evaluable for hematologic response. Major responses were seen in 80% of the 49 evaluable patients, with ten CRs (20%) and 29 PRs (59%). An additional nine patients (19%) had MRs. Only one evaluable patient has not responded to therapy.

Two patients had IFN dose escalations from 2×10^6 U/m² to 10×10^6 U/m² after 4 to 5 months of therapy. The IFN dose in the single nonresponder was increased after 4 months of IFN. There was no improvement over the next 2 months, and IFN therapy was discontinued. No further therapy was administered (except for transfusions) over the next 16 months. The other patient in whom the IFN dose was escalated had not responded despite 5 months of IFN, although his ANC had increased from 144 to 620 cells/ μ L. He showed further improvement over the next month but was not considered a PR until 1 month after stopping IFN treatment (at the end of a full year).

Only one patient had a worsening of his blood counts while receiving IFN. The patient initially responded (MR) to IFN and was randomized to an additional 6 months of IFN at the end of 1 year. After 14 months of therapy, his blood counts deteriorated, and IFN therapy was discontinued.

Changes in peripheral and bone marrow parameters during IFN therapy. As shown in Table 2, the median hemoglobin level improved continuously over the first 12 months of IFN therapy, increasing from 10.6 g/dL to 14.7 g/dL. The platelet count improved dramatically over the first 3 months, but there was only minimal improvement with subsequent therapy. The ANC also improved rapidly, but

Table 2. Changes in Peripheral Blood and Bone Marrow Parameters During IFN Therapy

Parameter	Month (n)					
	0 (53)	3 (53)	6 (50)	9 (42)	12 (34)	18 (9)*
Hb (g/dL)	10.6†	12.2 (.0001)‡	13.7 (.0001)	13.9 (.003)	14.7 (.02)	15.3
Plts (× 1,000 cells/μL)	95	254 (.0001)	240	249 (.06)	277 (.05)§	292
WBC (× 1,000 cells/μL)	3.6	3.8	4.4 (.005)	4.4	5.0 (.003)	6.3 (.05)
ANC (× 1,000 cells/μL)	0.7	1.8 (.0001)	2.0 (.009)	1.9	2.5 (.003)	2.9
BM cellularity (%)	78	50 (.0006)	47	34 (.01)	45 (.02)	48
BM hairy cells (%)	90	70 (.0001)	40 (.0009)	24	21 (.006)	10 (.08)
HCI	0.71	0.30 (.0001)	0.15 (.005)	0.07 (.001)	0.09	0.03 (.09)
NHCI	0.09	0.15 (.0001)	0.25	0.18	0.35 (.01)	0.44
NAP¶	204	100 (.0001)	77 (.0001)	31 (.003)	40	40

Abbreviations: Plts, platelets; BM, bone marrow.

*Due to randomization at 12 months to therapy v observation.

†Median.

‡P value v preceding time point by Wilcoxon paired signed-rank test (omitted if $P > .1$).

§P = .01 v 6 months.

||P = .06 v 9 months.

¶Normal range, 13 to 100.

there continued to be significant improvement over the first year.

Most of the bone marrow changes also occurred during the first 9 months. The bone marrow cellularity significantly decreased over the first 3 months (Table 2) and decreased further from months 6 to 9, prior to increasing slightly from months 9 to 12. The relative percentage of hairy cells in the bone marrow appeared to decrease continuously over the first year, with a further decrease ($P = .08$) from months 12 to 18 (nine patients only). The HCI significantly decreased over the first 9 months of IFN, with little subsequent change. The NHCI increased over the first 3 months but changed little over the next 6 months. However, there was significant improvement in the NHCI from months 9 to 12.

Table 2 also illustrates the dramatic changes that occur in the NAP with IFN therapy. The median NAP decreased from 204 to 31 over the first 9 months of IFN, but there was no further change over the next 3 to 9 months.

Toxicity. The nonhematologic toxicity that occurred in out HCL patients was very similar to that described in prior reports.^{6,13} Constitutional symptoms occurred in all patients and consisted of a "flu-like syndrome," fatigue, and anorexia. Gastrointestinal symptoms were common and included nausea/vomiting (17%) and diarrhea (32%). Neurological toxicity included both peripheral neuropathies (32%) and CNS dysfunction (21%). The latter usually consisted of subtle memory loss or depression, except for one patient with Cancer and Leukemia Group B (CALGB) grade 3 confusion that required a temporary withdrawal of IFN therapy. Other

common toxicities included injection site inflammation (45%), dry skin or seborrheic dermatitis (58%), alopecia (17%), and chemical hepatitis (74%). The latter was usually manifested as an elevated SGOT or SGPT level (79%), but the alkaline phosphatase value was occasionally elevated (28%). All toxicity was CALGB grade 1 or 2 (except for the one patient noted before), and no patient discontinued therapy due to toxicity, although one patient with severe fatigue refused randomization.

Transient myelosuppression was a very common event during the initial 1 to 2 months of therapy. Changes in the ANC were of the greatest magnitude (Table 3), but transfusions were occasionally required because of worsening anemia (two patients) and thrombocytopenia (two patients) due to IFN therapy.

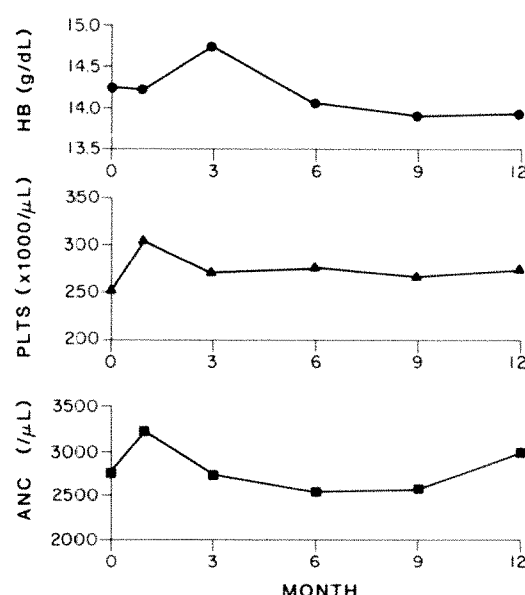


Fig 1. Changes in peripheral blood counts after stopping IFN therapy. The data represent the mean values at each time point. (HB, hemoglobin; PLTS, platelets).

Table 3. Transient Myelosuppression Due to IFN Therapy

Parameter	n*	Percent Decrease	Time to Nadir (wk)
Hemoglobin	33	8†	3
Platelets	36	25	1
ANC	47	54	2

*Patients were excluded from this analysis if they were transfusion dependent prior to IFN therapy, had no circulating neutrophils, or had an incomplete collection of blood counts.

†Median.

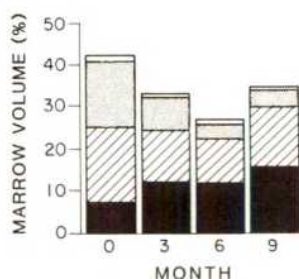


Fig 2. Changes in bone marrow subpopulations after stopping IFN therapy. The height of each bar represents the median volume of each subpopulation, as defined in Materials and Methods. The total height represents the marrow cellularity. ▨, myeloid; ▤, erythroid; ■, HCL; □, other.

Follow-up after discontinuation of IFN therapy. Of the 53 patients described in this report, 34 patients have completed at least 12 months of IFN therapy. Twenty-six patients were entered in the randomized study, 13 patients in each arm (12 v 18 months of IFN therapy). Eight patients were not randomized, mainly due to the fact that the randomized study was not opened until February 1985. Twenty-four patients have not been receiving IFN for at least 3 months and were studied further, as described in Materials and Methods. Sixteen patients discontinued IFN therapy after 12 months, seven patients completed 18 months of IFN treatment (6 months after randomization), and one patient discontinued IFN therapy after 6 months due to no response. This subset of 24 patients is fairly representative of the entire group of 53 patients: median age of 47 years, 88% male, and 75% complete or partial responders. The median time without IFN therapy is 8.5 months, with a maximum of 16 months. There was a significant increase during the first post-IFN month in the platelet count ($P = .004$) and ANC ($P = .05$), which reversed over the next 2 months (Fig 1). There was a significant increase in the hemoglobin level ($P = .01$) from months 1 to 3 after stopping IFN therapy, which reversed over the subsequent 3 months. In addition, the NAP

increased over the first 3 months in all 16 patients studied, with the median NAP increasing from 40 to 92 ($P < .001$). There were no other significant changes in any of the peripheral blood parameters over the first year of follow-up.

There were also definite changes in the bone marrow over the first 3 months after stopping IFN therapy (Fig 2). Sixteen patients had evaluable marrows both prior to stopping IFN treatment and 3 months later. (Five patients had severely hypocellular marrows at 3 months only, one patient had a severely hypocellular marrow both prior to stopping IFN therapy and 3 months later, one patient had a poorly prepared marrow at 3 months, and one patient did not have a marrow prior to stopping IFN treatment.) Over the first 3 post-IFN months there was a decrease (not statistically significant) in the median cellularity (42.5% v 35%, $P > .05$) and an increase in the relative proportion of hairy cells (21.5% v 47.5%, $P < .001$). The decrease in total cellularity appears to be due to a decrease in the myeloid (17.6% v 8.3%, $P < .001$) and erythroid (18.3% v 11.7%, $P < .01$) volumes, but there was no significant decrease in the myeloid:erythroid ratio (0.9 v 0.6, $P > .05$). An example of these morphological changes is presented in Fig 3. There were no statistically significant changes over the subsequent 9 months, although there was an overall increase in the leukemic volume.

A small subset of six patients did have a major increase in HCL over the first 9 months after IFN treatment (Fig 4). Although the follow-up has been short, only one patient has required further therapy after relapsing with a high WBC (23,000 cells/ μ L) after 6 months of observation. His initial response status was MR, and he has responded to his second course of IFN over the first month of treatment.

DISCUSSION

This paper confirms our preliminary report of the effectiveness of IFN in advanced HCL.⁵ IFN, when administered at a dose of 2×10^6 U/m² three times weekly, leads to normalization of blood counts and improvement in the bone

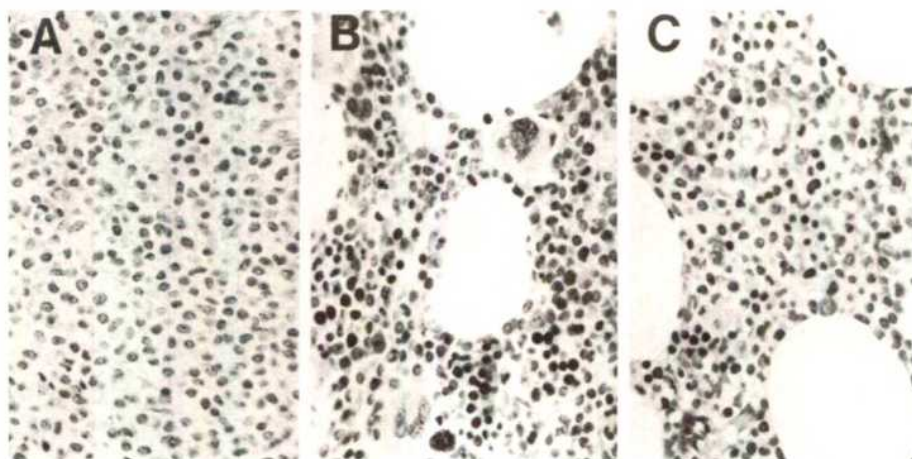


Fig 3. Bone marrow changes in a representative patient during and after IFN therapy. Prior to IFN therapy (A), the bone marrow was 95% cellular with 83% hairy cells. After 18 months of IFN therapy (B), the marrow was 75% cellular with less than 5% hairy cells, and the patient was considered to have a CR. However, there was myeloid hypoplasia with a myeloid:erythroid ratio of 0.7. Three months after stopping IFN therapy (C), the cellularity was 50% with 25% hairy cells. Note the further decrease in the myeloid:erythroid ratio to 0.5.

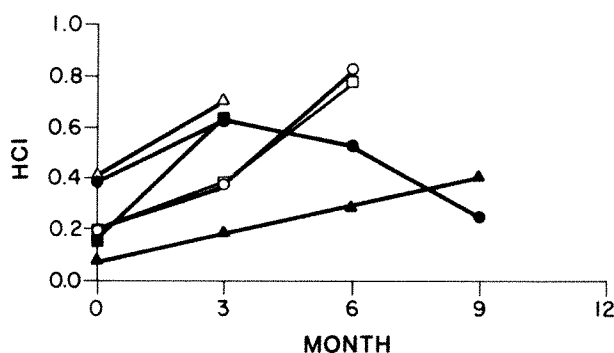


Fig 4. Six patients with an increased HCL (>0.4) after stopping IFN therapy. Only one patient (○) has required further IFN therapy. Note also that one patient (●) had an increase in the HCL from month 0 to 3, which decreased over the next 6 months.

marrow in 80% of patients treated for at least 6 months. The therapy is well tolerated, with no patients discontinuing IFN treatment due to toxicity. Comparable results have been reported with the similar drug, interferon alfa-2a.^{10,14,15} Changes in the peripheral blood counts and bone marrow occur rapidly, with significant improvement over the first 3 months, especially in the platelet count. There continues to be improvement over the first year in both the peripheral blood counts and bone marrow.

One of the primary purposes of this analysis was to define the extent and course of hematologic changes occurring after discontinuing IFN therapy. We demonstrate that responses to IFN are quite durable, with only one of 24 patients followed for a median of 8.5 months who required further therapy. In addition, there were no significant changes in peripheral blood counts over the first post-IFN year, except for a rebound increase in all three cell lines demonstrated at months 1 to 3. There did appear to be significant changes in the bone marrow after stopping IFN therapy that were characterized by a decrease in normal myeloid and erythroid elements. In addition, there was a trend towards an increasing HCL over the first year after stopping IFN treatment, which has not yet reached statistical significance.

Study of the changes in the bone marrow after stopping IFN therapy may provide insight into the mechanisms of interferon-induced myelosuppression. The major hematologic toxicity of interferons is leukopenia, with a decrease of 40% to 60% in the WBC during the first week of treatment but with no further decrease with continued therapy.¹³ Ernstoff and Kirkwood¹⁶ have previously analyzed bone marrows in patients with nonhematologic malignancies who received IFN. There was a trend toward an increase in normal precursors but no maturation arrest. Our data demonstrate that there is a marked decrease in normal myeloid and erythroid marrow elements after stopping IFN therapy

simultaneous with a rebound increase in the peripheral blood count. This suggests that IFN directly or indirectly inhibits the release of normal marrow elements and that this process is reversed when IFN therapy is discontinued—a hypothesis previously proposed by Ernstoff and Kirkwood.¹⁶

We also demonstrate in this paper that IFN leads to normalization of another hematologic parameter that is abnormal in patients with HCL, the NAP. A previous report by Golomb et al⁸ noted an increased NAP in 15 of 17 patients studied. The median pretreatment NAP in our patients was markedly elevated at 204, which decreased to a nadir of 31 by month 9 but increased after stopping IFN therapy. A high NAP score may reflect a decrease in marrow myeloid elements, since these were the two major changes after discontinuing IFN treatment. A more detailed analysis of the relationship of NAP to peripheral blood counts and bone marrow subpopulations is in progress.

We continue to emphasize that IFN therapy is only palliative because discernible hairy cells remain in the bone marrow of virtually all patients. In addition, the bone marrow remains abnormal, even in complete responders, with increased reticulin and myeloid hypoplasia.⁹

We propose that patients with progressive HCL following splenectomy be treated with IFN for at least 6 months. Responding patients should be treated for 1 year. Information regarding longer periods of treatment will be available after analysis of a multiinstitution study randomizing patients to 12 v 18 months of IFN therapy. Practicing physicians must be aware of the transient myelosuppression that occurs in the first 1 to 2 months of therapy, possibly precipitating the need for RBC and/or platelet transfusions. Following the discontinuation of IFN therapy patients should be monitored with complete blood counts every 1 to 3 months. Although the bone marrow may change significantly after IFN treatment is stopped, it should not necessarily be used as a guide to reinstitution of IFN therapy.

We cannot yet recommend IFN as initial therapy to patients in lieu of splenectomy, although Quesada and co-workers recently report the attainment of a CR in five of seven nonsplenectomized patients.¹⁷ In addition, the eventual role of IFN in the therapy of HCL is uncertain because of the high complete response rates (23% to 65%) reported from preliminary studies of the adenosine deaminase inhibitor pentostatin.¹⁸⁻²² If possible, patients with HCL should continue to be entered in clinical trials.

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The Secretory Pathway of Bovine Platelets

By James G. White

Human platelets contain tortuous channels in their cytoplasm, the surface-connected or open canalicular system (OCS), that communicate directly with the surrounding medium through openings on the surface membrane. Some workers have suggested that the OCS serves as the egress route for products secreted during the release reaction. Others have proposed alternate secretory pathways. Since bovine platelets lack the OCS found in human cells, the present study has examined the secretory mechanism of these cells to see whether it can shed light on the mystery of human platelet secretion. Bovine platelet granules, in contrast to human granules, are located more peripherally in resting cells (often in contact with the plasma membrane), most do not move centrally following thrombin stimulation as do human platelet granules, and many fuse directly with the external plasma membrane without any intermediate channel. The lack of peripheral location of human granules, their central rather than peripheral move-

ment during secretion, and the presence of extensive channels are all consistent with the larger importance of the secretory channel to human platelets. Thus, though studies of bovine secretion do show that platelets can secrete their granules by direct fusion of granule and surface membranes, other differences from human platelets emphasize that this pathway, although important to bovine platelet secretion, is less important in human platelets. Studies of bovine platelets also show that the OCS is more dynamic than might have been considered from human studies and can form rapidly in response to stimulation. Such newly formed channels are used as a conduit for secretion of granule contents. The finding emphasizes the importance of channels for granule secretion in platelets generally and puts a new perspective on the ability of these cells to form channels rapidly in response to stimulation.

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HUMAN PLATELETS are secretory cells.¹ Three different types of secretory organelles including alpha granules, dense bodies, and lysosomes are randomly dispersed in the cytoplasm of resting platelets.²⁻⁴ Following exposure to aggregating agents, platelets lose their discoid form, become irregular with multiple pseudopods, and concentrate organelles in cell centers.⁵ The physical alterations are intimately associated with the discharge of chemical substances to the surrounding medium.⁶

The mechanism by which products stored in the secretory granules of human platelets are released to the exterior without simultaneous loss of cytoplasmic constituents has been the subject of considerable interest.⁷⁻¹² Investigations using electron-dense tracers revealed the existence of a surface-connected open canalicular system (OCS) in resting human cells.¹³ After activation, storage organelles appeared to fuse with channels of the OCS, which then served as conduits for the release of secretory products.¹²⁻¹⁵ Other workers using different techniques proposed an alternate pathway for secretion.¹⁶ They suggested that centrally concentrated organelles fuse to form a closed sac that then migrates to the platelet surface, attaches to the membrane, and extrudes its products to the outside.

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A recent study of bovine platelets has suggested the existence of a model for examining the two different concepts of platelet secretion.¹⁷ Zucker-Franklin et al¹⁷ have shown that bovine cells lack the OCS found in human cells. Therefore, cattle platelets must have some mechanism other than channels of the OCS for discharging products from secretory granules to the outside.¹⁸ The present study has searched for the secretory pathway in bovine platelets with the aid of freeze-fracture and electron-dense tracers.

MATERIALS AND METHODS

Blood for the present study was obtained from healthy adult cattle housed under direct supervision of the School of Veterinary Sciences at the University of Minnesota. Samples aspirated from the external jugular vein were mixed immediately with citrate-citric acid-dextrose (CCD) (93 mmol/L trisodium citrate, 70 mmol/L citric acid, 140 mmol/L dextrose, pH 6.5) in a ratio of nine parts blood to one part anticoagulant.¹⁹ Blood was sedimented at room temperature under a force of 200 g for 20 minutes. Platelet-rich plasma (C-PRP) above the buffy coat was aspirated and transferred to separate tubes. After determining the platelet count, the samples of C-PRP in each tube were combined with equal volumes of the CCD anticoagulant and centrifuged to pellets. The plasma was replaced with Hanks' balanced salt solution (HBSS) containing 0.1 mmol/L EDTA. After 30 minutes at 37 °C, the pellets were resuspended in HBSS with or without EDTA to produce a platelet count of 500,000/ μ L and returned to the 37 °C water bath.²⁰ Samples were fixed for study in the electron microscope at 30 seconds and 1, 3, and 5 minutes after exposure to thrombin at concentrations of 1, 3, and 5 U/mL. Each thrombin concentration and time of exposure was examined in four to seven experiments.

Fixation was accomplished by combining the sample at the appropriate time with an equal volume of 0.1% glutaraldehyde in White's saline (a 10% solution of a 1:1 mixture of (1) 2.4 mmol/L NaCl, 0.1 mmol/L KCH₃, 46 mmol/L MgSO₄, and 64 mmol/L Ca (NO₃)₂ · 4H₂O and (2) 0.13 mol/L NaHCO₃, 8.4 mmol/L NaH₂PO₄, and 0.1 g/L of phenol red, pH 7.4). After 15 minutes the samples were centrifuged to pellets and the supernatant fixative removed and replaced with 3% glutaraldehyde in the same buffer.¹⁹ The samples resuspended in the second aldehyde fixative were maintained at 4 °C for 30 minutes and then sedimented to pellets. The supernatant was removed and replaced with either 1% osmic

acid in Zetterquist's buffer or 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for one hour at 4 °C.²¹

Tannic acid has been used in our previous studies as an electron-dense stain for the OCS of human platelets.^{22,23} In the present study the method was modified from the procedure reported by Somlyo²⁴ to stain the secretory pathway of bovine platelets. Bovine cells washed and resuspended as described before and exposed to thrombin in the presence or absence of EDTA were fixed through our usual sequence of 0.1% glutaraldehyde in White's saline followed by sedimentation to a pellet, removal of the supernatant, and replacement with 3% glutaraldehyde in the same buffer for two hours at 4 °C. After completion of initial fixation steps, the pellets were washed three times and stored overnight in 0.1 mmol/L cacodylate buffer, pH 7.2. The buffer was decanted the following day and replaced with 4% tannic acid in 0.1 mmol/L cacodylate buffer, pH 7.2, for four hours at room temperature. The supernatant was decanted, the pellets washed in cacodylate buffer, and the samples postfixed in 2% osmic acid alone or combined with 1.5% potassium ferrocyanide for two hours at 4 °C.

All samples were dehydrated in a graded series of alcohols and embedded in Epon 812. Thin sections cut from the plastic blocks on an ultramicrotome were examined unstained or after staining with uranyl acetate and lead citrate to enhance the contrast. All examinations were made in a Phillips 301 electron microscope.

Some of the aldehyde-fixed control and activated platelet samples from each experiment were combined with glycerol, frozen in liquid nitrogen, and freeze-fractured under high vacuum.²⁵ Carbon platinum replicas prepared from exposed surfaces of the fractured platelet pellets were cleaned in detergent and examined in the transmission electron microscope.

RESULTS

Resting Bovine Platelets

The fine structure of bovine platelets was described previously by Myers et al¹⁸ and by Zucker-Franklin and her colleagues.¹⁷ Bovine platelets are similar to human cells in many respects. Like platelets of humans, cattle platelets are discoid in form, and their lentiform appearance is supported by a circumferential microtubule (Figs 1 and 2). Bovine platelets contain the granules, dense bodies, and microchondria that are also found in human cells.

There are, however, significant differences between human and bovine platelets. Cattle cells are slightly smaller, contain fewer dense bodies, and their cytoplasm is dominated by granules two to three times larger than those in human platelets.^{17,18} Many of the large granules are spindle shaped and often appear to contact the inside of the surface membrane (Fig 2). An extensive network of tortuous channels communicating with the cell surface and referred to as the OCS in human platelets^{23,26} was not found in bovine cells (Figs 3 and 4). This confirmed the work of others showing that cationized ferritin, an electron-dense tracer, easily entered the channel system of human platelets but did not appear in bovine platelet cytoplasm.¹⁷ Replicas of freeze-fractured bovine platelets also failed to reveal the typical pits formed by channels of the OCS communicating with the surface of platelets from humans (Fig 3).

Thin sections of resting platelets prepared by our usual fixation procedures in the present study also failed to reveal the well-defined system of channels evident in platelets from

humans (Figs 1 and 2). Bovine platelets stained with tannic acid lacked an OCS, and replicas of freeze-fractured cattle platelets contained only rare dimples or pits comparable to the openings of the OCS on the surface membrane of human cells until after stimulation by thrombin (Figs 3 to 6). However, small protrusions about 0.2 μ m in diameter and devoid of intramembranous particles were frequently present on the P face of freeze-fractured resting bovine cells (Fig 3). Fractures through the membrane into the cytoplasm often revealed contact between membranes of the large granules and the undersurface of the cell wall at sites resembling the protrusions or indentations seen on the P face (Fig 4).

Elements of a second system of channels, the dense tubular system (DTS), were fully developed in bovine platelets. However, membrane complexes formed by the interaction of canaliculi from the OCS and DTS were absent (Figs 1 and 2).²² The presence of the platelet-specific peroxidase²⁶ localized to the DTS channels of human platelets confirmed the presence of a DTS in bovine platelets. Thus cattle cells contain the DTS but are deficient in the specialized membrane complexes found in human cells and in platelets from other species.

Thrombin-Stimulated Bovine Platelets

Aggregates. Thin sections of aggregated bovine platelets bore a superficial resemblance to human platelet thrombi. Shape change was evident, and the groups of cells were closely molded to each other as in hemostatic plugs. Dense spots resembling the contracted masses of actomyosin present in thrombin-stimulated human cells were present in many aggregated bovine platelets. However, the central concentration of organelles and enclosure within constricted rings of microtubules and microfilaments, regularly present in activated human platelets,⁵ were rarely evident in bovine platelets. Instead, the granules remained dispersed near the cell periphery. Tannic acid staining of the bovine platelet aggregates revealed electron-dense tracer outlining the spaces between aggregated platelets (Figs 7 and 8). In some cells the tannic acid-osmium product appeared in channel-like structures penetrating into the cytoplasm of the clumped platelets close to the dense spots of actomyosin. Some granules were also stained by tannic acid-osmium, which indicated they were connected to the cell wall or channels reaching the platelet surfaces.

Activated platelets. Thin sections of thrombin-stimulated but not aggregated bovine platelets revealed additional differences in their response compared with their human counterpart (Figs 9 to 20). Spikelike pseudopods extending from shape-changed bovine platelets were thinner and straighter than those formed on activated human platelets. Occasional microtubules are evident in human platelet pseudopods, but most surface projections lack the polymer. Almost every pseudopod extending from bovine platelets contained one or several microtubules interspersed with microfilaments. Microtubules were also present in the cytoplasm of activated bovine platelets (Fig 9). However, instead of being organized in constricted rings around centrally concentrated organelles, the tubules were irregularly dispersed in bundles or loose arrangements. The granules in

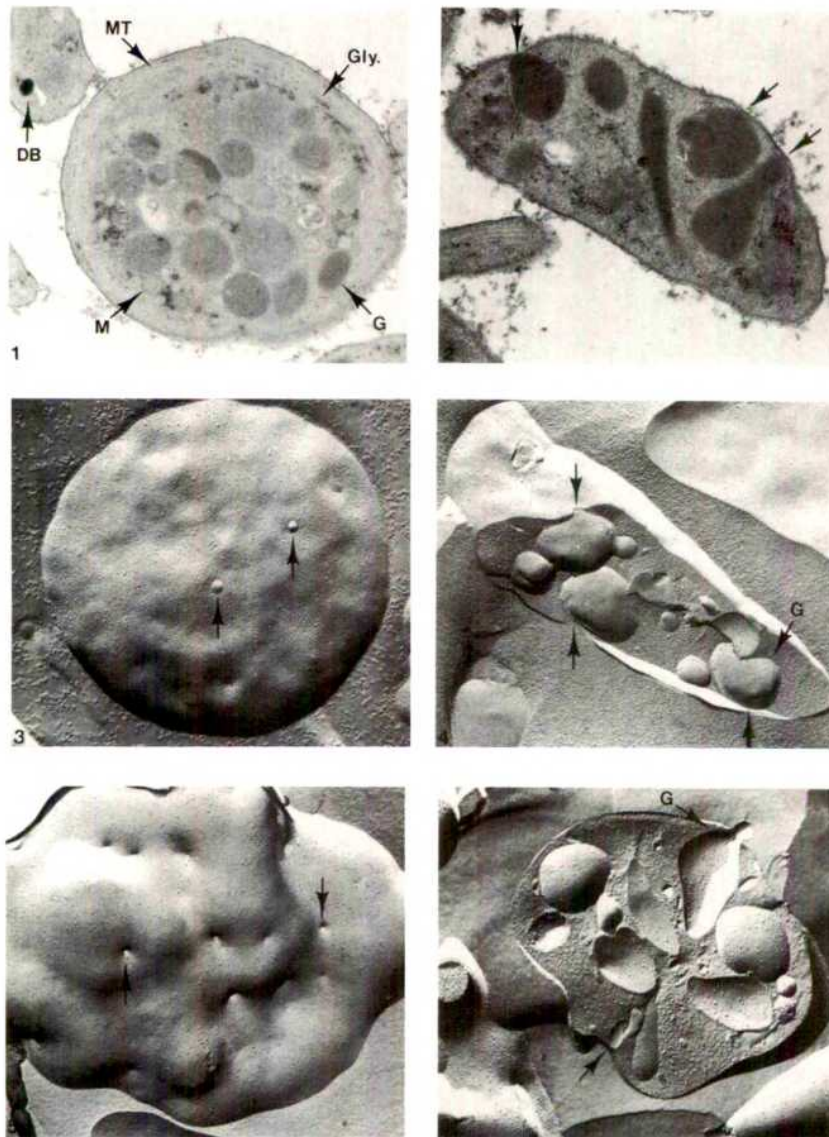


Fig 1. Resting bovine platelet. A circumferential microtubule (MT) coiled just under the surface membrane supports the lentiform appearance of the unstimulated cell. Granules (G), occasional mitochondria (M) and a few dense bodies (DB) together with glycogen (Gly) particles are randomly dispersed in the cytoplasm. The resting cell does not appear to contain channels of the open canalicular OCS or dense tubular systems (DTS). (Original magnification $\times 24,000$.) Fig 2. Cross section of discoid cattle platelet. Granules are significantly larger than those in human platelets. They often appear to be in close association ($\uparrow$) or contact with the platelet membrane. (Original magnification $\times 28,000$.) Fig 3. Replica of freeze-fractured bovine platelet. The surface membrane of a discoid cell has been split by the procedure, thereby revealing the outside of the inner layer, or "P" face. Indentations representing sites of continuity between channels of the open canalicular and the surface membrane in human cells are rarely seen in replicas of resting bovine platelets. Dimples ($\uparrow$) or protrusions free of the intramembranous particles covering the rest of the exposed surface are evident on most of the replicas. They may represent sites of contact between granules in the cytoplasm and the inner surface of the cell membrane. (Original magnification $\times 22,000$.) Fig 4. Replica of discoid bovine platelet. Granules (G) in the cytoplasm are in close proximity to depressions in the surface membrane ($\uparrow$) at several points. (Original magnification $\times 28,000$.) Fig 5. Replica of washed bovine platelet exposed to thrombin (3 U/mL, five minutes) in the presence of EDTA. The "P" face contains several indentations ($\uparrow$) similar, if not identical, to the openings of the OCS on replicas of resting human platelets. (Original magnification $\times 25,000$.) Fig 6. Thrombin-activated bovine platelet (1 U/mL, three minutes). Shape change is under way in this cell, but the granules remain peripherally oriented. One organelle (G) has caused the surface membrane to protrude. This may precede extrusion of organelle contents. A small channel ($\uparrow$) is also apparent close to the cell membrane in this cell. (Original magnification $\times 28,000$.)

most platelets were peripherally oriented near the cell membrane rather than concentrated in cell centers.

Although tannic acid failed to stain channels of the OCS or granules in resting bovine platelets, it did so in thrombin-activated cells. Tannic acid appeared to react selectively with

some product in platelet granules, which resulted in a heavy deposit of osmium black (Figs 7 to 12). The electron-dense stain appeared to reach granules through connections between the organelles and the cell surface. The mechanism for development of the openings permitting penetration of

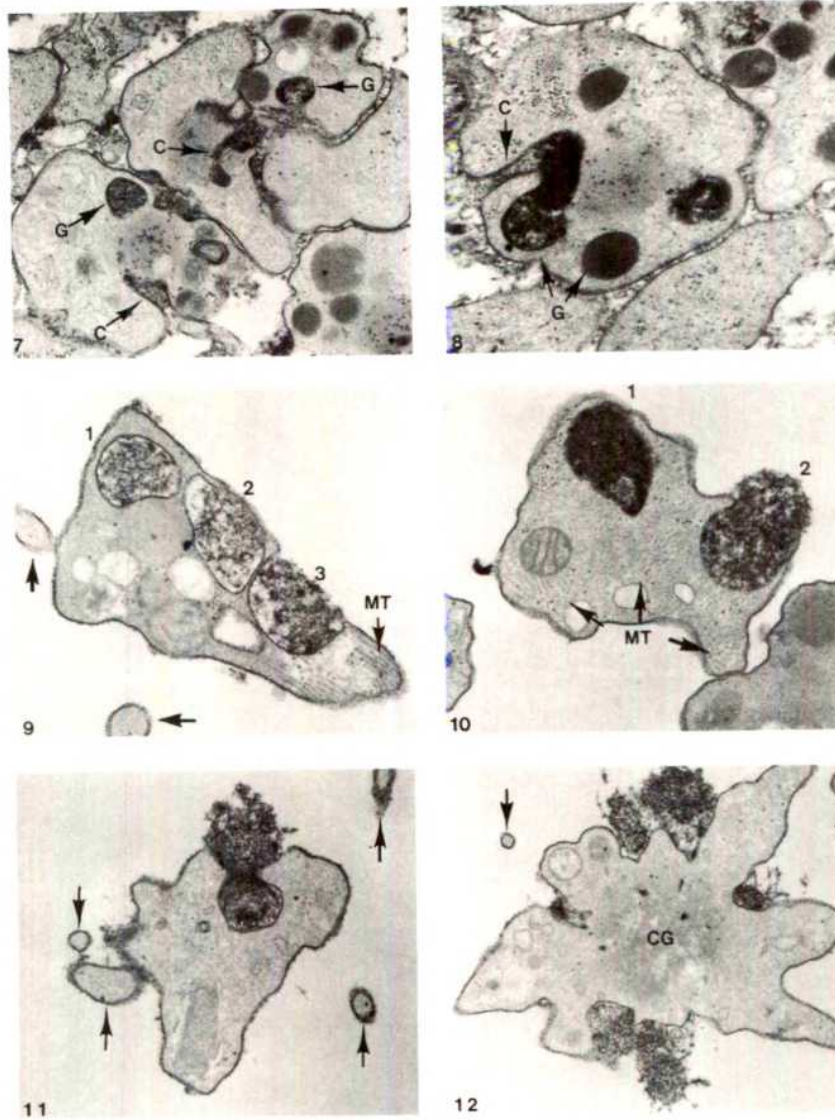


Fig 7. Thrombin-aggregated platelets stained during fixation with tannic acid. Osmic acid deposited as an electron-dense stain by tannic acid coats the cell surface, fills channels penetrating into the cells, and has entered some granules (G) similar in size to unstained organelles. Two of them are in direct continuity with the cell surface through stain-filled channels (C). (Original magnification $\times 17,000$.) Fig 8. Thrombin-aggregated bovine platelets. The preparation is the same as that shown in Fig 7. Tannic acid coats the surface of clumped platelets and fills a channel (C) penetrating into one of the cells. One granule (G) is in direct continuity with the channel, and another adjacent to it is unstained. (Original magnification $\times 28,000$.) Fig 9. Thrombin-activated platelet (1 U/mL, three minutes). Little evidence of shape change is apparent in this cell. Microtubules are still evident at the platelet periphery and adjacent pseudopods. One granule (1) stained by tannic acid appears unconnected to the cell surface, another organelle (2) is very close to it, and a third (3) is open to the surrounding medium. Serial sections of this cell (not shown) reveal that organelles 1 and 2 are also directly connected to the platelet surface. (Original magnification $\times 34,000$.) Fig 10. Thrombin-activated platelets (5 U/mL, one minute). The microtubules (MT) in this cell are in three separate groups. One granule (1) is filled with stain and appears unconnected to the cell surface. However, it must be connected above or below the plane of section to the surface membrane in order to take up tannic acid. Another granule (2) is connected to the cell surface, and its contents appear to be in the process of extrusion. (Original magnification $\times 30,000$.) Fig 11. Thrombin-activated platelet (5 U/mL, one minute). Every pseudopod ($\uparrow$) extending from this platelet or adjacent cells contains microtubules. A granule filled with tannic acid-stained material appears to be in the process of discharge from the platelet. (Original magnification $\times 30,000$.) Fig 12. Thrombin-activated platelet. This cell appears to have reacted maximally to thrombin stimulation. All of the granules appear to have fused with the cell surface and discharged their contents to the exterior. Tannic acid-stained material most likely represents granule contents fixed in situ during extrusion. The dense spot remaining in the cell center is a contracted gel (CG) of actomyosin. Microtubules fill adjacent pseudopods ($\uparrow$). (Original magnification $\times 24,000$.)

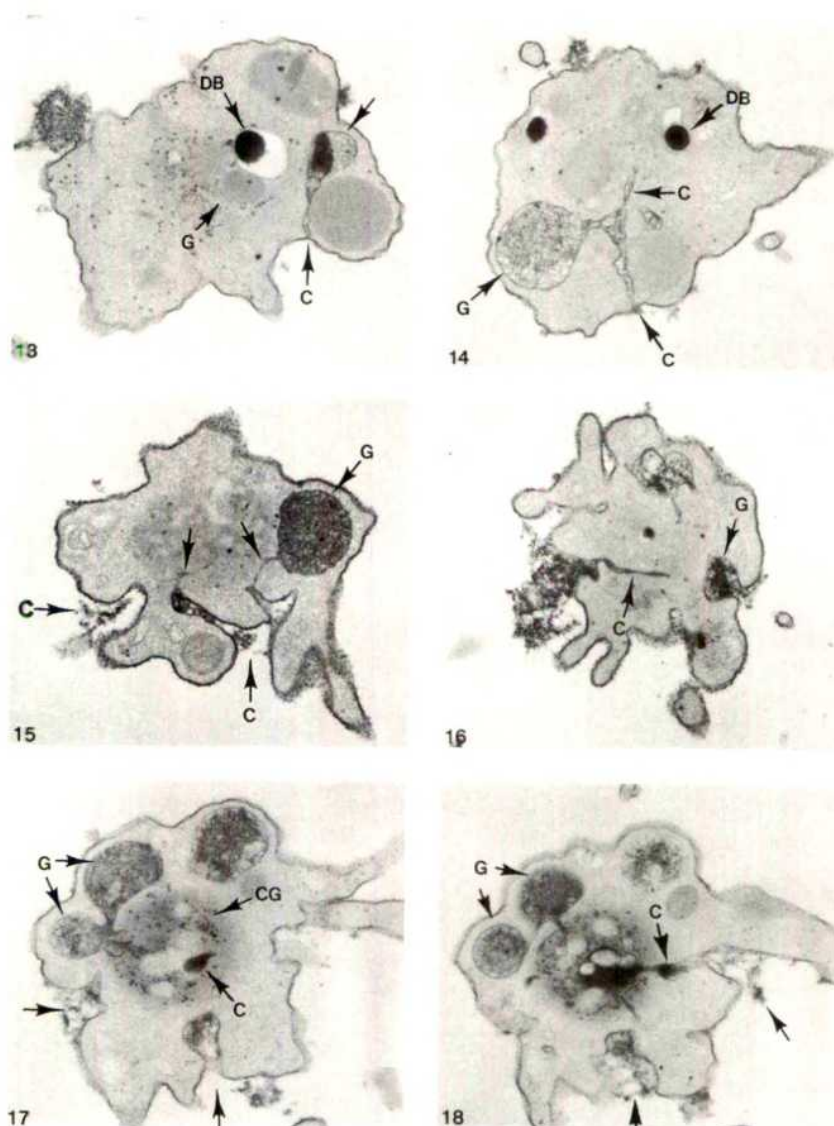


Fig 13. Thrombin-activated platelet (1 U/mL, five minutes). The cell contains small and large unstained granules (G) and a dense body in its cytoplasm. A small granule (†) stained with tannic acid is in direct continuity with a fragile-appearing channel (C) connected to the surface membrane. (Original magnification $\times 28,000$.) Fig 14. Thrombin-activated platelet (3 U/mL, five minutes). Dense bodies (DB) and unstained small and large granules fill the cell cytoplasm. A large granule heavily stained by tannic acid is in direct continuity with a channel (C) that undergoes branching. A channel burrows deeper into the platelet cytoplasm, and the other is connected to the cell surface. (Original magnification $\times 28,000$.) Fig 15. Thrombin-activated platelet (5 U/mL, one minute). Several channels (C) or empty granules have scalloped the cell membrane. Fine canaliculi (†) stained by tannic acid extend from the surface deeper into the cytoplasm. One of them connects to a stain-filled granule (G). (Original magnification $\times 28,000$.) Fig 16. Thrombin-activated platelet (5 U/mL, one minute). The cell in this example is irregular with many pseudopods. A granule communicates with the cell membrane on one side of the cell and discharges its contents to the exterior. A channel (C) from deep in the cytoplasm communicates with the surface on the other side. Tannic acid-stained material fills the channel and has been fixed in the process of extrusion from the cell. (Original magnification $\times 28,000$.) Figs 17 and 18. Serial sections of a thrombin-activated platelet. The cell has at least two sites (†) on the surface where tannic acid-stained granule material is in the process of discharge. Two of the tannic acid-stained granules (G) appear to communicate with a channel at the edge of the contracted gel (CG) of actomyosin. A small fragment of a channel (C) filled with tannic acid is buried in the contractile gel in Fig 17 and extends to the cell surface in Fig 18. (Original magnifications $\times 26,000$, Fig 17; $\times 26,000$, Fig 18.)

tannic acid into granules was not clear. However, it must be related to the action of thrombin because connections were not evident in control platelets. The discharge of stained granule contents from thrombin-activated platelets appeared to follow two routes. Most of the granules close to the surface fused with the membrane and secreted directly to the outside medium (Figs 9 to 12). Some examples suggest that granule

products are actively extruded (Figs 10 to 12). Internal contraction leading to the formation of dense spots of contractile gel in thrombin-activated bovine platelets may provide the force for expulsion (Fig 12).

In addition to demonstrating the interaction between granules and the surface membrane in thrombin-activated bovine platelets, tannic acid staining also revealed the devel-

opment of a primitive channel system in activated cells (Figs 13 to 20). The suggestion that channels were newly developed came from observations made on replicas of freeze-fractured platelets. Apertures of the OCS onto the surface membrane of resting and activated human platelets were rarely seen on discoid bovine cells (Fig 3). They were regularly present, however, following exposure of bovine platelets to thrombin (Fig 5). In thin sections of tannic acid-stained bovine platelets, electron-dense product penetrated into fragile channels extending for variable distances into the cytoplasm. Communication between stain-filled channels and granules was easily identified. The channels appeared fragile and lacked the tortuosity and extensive distribution of the OCS in human platelets (Fig 20). Channels were also apparent in replicas of thrombin-stimulated bovine platelets (Figs 6 and 21 to 24). Some branching was noted in replicas, but, in general, the channels were very simple structures.

DISCUSSION

The present investigation has used ultrastructural techniques to search for the secretory pathway in bovine platelets. Cattle cells resemble human platelets closely.^{17,18} Resting platelets from both species have a characteristic discoid shape supported by a circumferential microtubule. The cytoplasm of human and bovine platelets is filled with secretory organelles including dense bodies and alpha granules. Mitochondria and glycogen particles are common in both cells. There are significant differences, however.^{17,18} Bovine platelets are slightly smaller than human cells and more elliptical in form. Dense bodies are less frequent, and the granules are two to three times larger than comparable organelles in human platelets. Granules in cattle platelets often appear to impinge on the surface membrane, whereas this close relationship does not occur in cells from humans.^{14,15}

The major difference between human and bovine cells lies in the membrane systems. An extensive system of channels communicating with the surface membrane and with each other is a characteristic feature of human platelets.^{23,27-29} Zucker-Franklin and her colleagues used an electron-dense tracer, cationized ferritin, and freeze-fracture to demonstrate that bovine platelets lack the surface-connected OCS.¹⁷ Examination of tannic acid-stained platelets and replicas of freeze-etched cells in this study confirmed the absence of a definitive OCS in resting cells from cattle.

The findings suggested that the bovine platelet could serve as a useful model to resolve conflicting concepts regarding the mechanism of platelet secretion. A search for the secretory pathway of activated human platelets had shown that granules fuse with channels of the OCS and are extruded from the cell through these conduits during platelet shape change and internal contraction.^{5,12,13} Other workers reached a different conclusion. Ginsberg et al¹⁶ used immunocytochemistry and electron microscopy to follow secretion in human cells. Centrally concentrated organelles in stimulated platelets fused together to form closed sacs. The sacs of secretory products migrated to the platelet surface, attached

to the membrane, and extruded their contents to the outside.

Since bovine platelets lack the OCS, the concept of secretion in which granules fuse with channels and use the canaliculi for discharge of products did not seem reasonable for cattle cells. Some variation of the hypothesis proposed by Ginsberg and colleagues¹⁶ would seem more tenable. However, the movement of granules toward platelet centers that precedes their intimate association and possible fusion into closed sacs occurs infrequently in bovine platelets.^{17,18}

In the present study thrombin-induced aggregation and activation of bovine cells caused shape change, pseudopod formation, and the development of stickiness, responses closely resembling those of human cells. The internal reaction of cattle platelets, however, was quite different. Central movement of randomly dispersed organelles and their inclusion within tight-fitting rings of microtubules and microfilaments, a regular feature of stimulated human cells,^{5,14,15} occurred rarely in bovine platelets. Instead the microtubule coil appeared to remain peripherally located or fractured during shape change, which resulted in random bundles in the cytoplasm and one to several polymers in each spikelike pseudopod. Granules remained dispersed and close to the inside surface of the cell membrane in thrombin-activated and -aggregated bovine platelets.

Examination of freeze-fractured bovine platelets after exposure to thrombin revealed a marked change from resting cells. Dimples indicating sites of communication between channels of the OCS and the surface were virtually absent from discoid platelets.¹⁷ However, pinpoint depressions, slightly smaller than the openings of the OCS on human platelets, were present on nearly every activated bovine platelet. Short invaginations resembling channels communicating with the surface membrane were present in the cytoplasm of freeze-fractured bovine platelets prepared after exposure to thrombin.

Tannic acid staining of thrombin-aggregated and -activated platelets also revealed dramatic changes from the appearance of resting bovine platelets. Electron-dense stain was deposited in many organelles with the size and shape of granules in activated cells. Some of these were in direct continuity with the irregular platelet surface. Other stained organelles were connected to the cell wall and to each other by narrow channels filled with electron-dense material. In many examples the stain appeared to catch granule products fixed during the process of extrusion from thrombin-stimulated bovine platelets.

The close relationship between the large granules and the surface membrane of bovine platelets would seem to obviate the need for OCS channels found in platelets from humans, pigs, rats, whales, horses, rabbits, and other species. It is surprising, therefore, that bovine platelets either have a few elements of OCS invisible in resting cells or develop them during shape change and internal contraction. Electron-dense stain entered channels communicating with the surface in activated platelets. The channels were narrow, compared with elements of the OCS in human cells, and usually short and unbranched. Some, however, extended far into platelet cytoplasm to form a direct connection to stain-filled

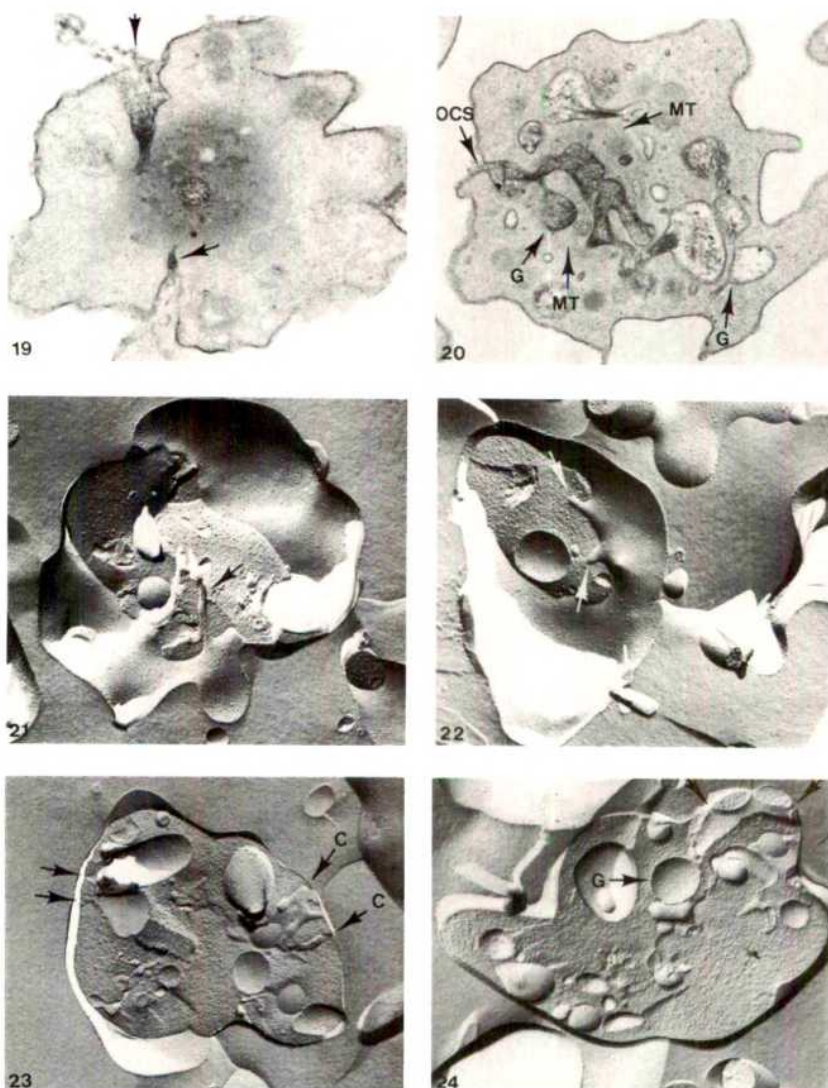


Fig 19. Thrombin-activated platelet (5 U/mL, five minutes). A fully transformed platelet is evident in this illustration. Shape change and contraction of actomyosin gel into a dense spot are advanced. At least two channels contain tannic acid-stained residue, which suggests that they are granules or carry granule products to the surface. (Original magnification $\times 25,000$.) Fig 20. Thrombin-activated human platelet (3 U/mL, three minutes). This cell has been exposed to the same conditions as bovine cells in the present study. A channel of the OCS in continuity with the surface membrane is dilated by tannic acid-stained material. The tortuous channels are connected directly to at least two granules (G), which are also stained by tannic acid. The constricted microtubule (MT) coils are cut in cross section in this example. (Original magnification $\times 23,000$.) Fig 21. Replica of thrombin-activated bovine platelet (3 U/mL, one minute). A fine channel ($\uparrow$) extends from the surface of the stimulated cell deep into the cytoplasm. (Original magnification $\times 28,000$.) Fig 22. Replica of thrombin-activated platelet (3 U/mL, one minute). Two short channels ($\uparrow$) are evidently extending inward from the surface membrane of the activated cell. (Original magnification $\times 30,000$.) Fig 23. Replica of thrombin-activated platelet (3 U/mL, one minute). Two small channels ($\uparrow$) appear to connect granules to the cell surface on the left. On the right, a channel (C) with two openings on the surface may provide a pathway for secretion of some granule products. (Original magnification $\times 28,000$.) Fig 24. Replica of thrombin-activated platelet. One edge of this cell has three openings ($\uparrow$) into a single channel of the OCS, which extends deeper into the cytoplasm close to a granule (G). (Original magnification $\times 30,000$.)

granules. It is possible that the channels developed in activated platelets were the result of infoldings or creases in the membrane or a collapse of granule walls following secretion of their contents. However, except for the extensive distribution of the OCS in human cells and the tortuosity and interconnection of the canaliculi, the appearance of the channels and relationship to granules and the cell membrane in bovine platelets were quite similar.

Thus, the difference in the mechanism of secretion in

bovine compared with human cells is not as great as it might at first seem. Bovine platelets do not have a well-defined OCS. As a result, they use the surface membrane as the primary route for discharge of products from secretory granules. Human platelets have an extensive system of internalized surface membrane formed into channels of the OCS. It is used as the primary route of secretion in human cells. Bovine platelets can develop primitive canaliculi following activation, and granule products may leave the cell

through these conduits. Yet it uses the cell surface as a preferential route for exocytosis. The basis for the species variations in platelet structure that result in different preferred routes for secretion of granule products remains unknown.

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Expression of the Human Monocyte Membrane Antigen gp55 by Murine Fibroblasts After DNA-Mediated Gene Transfer

By Richard A. Ashmun, Stephen C. Peiper, Matthew B. Rebutisch, and A. Thomas Look

Human DNA sequences that contain the gene encoding gp55, a cell surface glycoprotein expressed exclusively on mature human monocytes and monocytic leukemia cells, were isolated in a mouse genetic background. DNA from mature human monocytes was cotransfected with DNA from a molecularly cloned feline sarcoma virus containing the *v-fms* oncogene into NIH-3T3 cells. Transformed mouse fibroblasts that expressed gp55, based on their reactivity with the MY4, B44.1, or LeuM3 monoclonal antibodies, were selected by fluorescence-activated cell sorting. Regardless of which antibody was used for selection, equivalent binding of all three antibodies was observed for positive transformants. Secondary and tertiary mouse cell transformants were obtained after additional rounds of transfection and cell sorting with the use of DNA from primary and then secondary transformants. Southern blot analysis of the cellular DNA from two inde-

pendently derived tertiary subclones revealed a limited complement of human sequences, thus indicating that the gene encoding gp55 is included in fewer than 50 kilobases of human DNA. Independently derived tertiary subclones displayed concordant patterns of reactivity with 13 monocyte-specific monoclonal antibodies, thus indicating that each recognized an epitope on the product (gp55) of a single human gene. The 55-kilodalton cell surface polypeptide was specifically immunoprecipitated with a representative monoclonal antibody, 26if, from lysates of enzymatically radioiodinated peripheral blood monocytes and tertiary transformants. We conclude that gp55 is highly immunogenic and that a large number of independently derived monoclonal antibodies specific for human monocytes react with epitopes on this one molecule.

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MANY MONOCLONAL ANTIBODIES recognize epitopes on the surface of mature cells of the monocyte-macrophage lineage¹ and therefore have proved valuable for studying normal mononuclear phagocytes and their malignant counterparts. With few exceptions, the functions of cell surface antigens recognized by these reagents are unknown, but it is reasonable to speculate that such molecules participate in key cellular activities mediated by monocytes such as antigen processing or cell-mediated cytotoxicity.

One of the monocyte-specific monoclonal antibodies, MY4, precipitates a 55-kilodalton (kd) cell surface glycoprotein (gp55) that is present on monocytes, macrophages, and cells from patients with acute monocytic or myelomonocytic leukemia.²⁻⁴ At least two other monoclonal antibodies including Mo2⁵⁻⁷ and AML-2-23⁸⁻¹¹ display similar patterns of reactivity and immunoprecipitate proteins with similar apparent molecular weights. None of these antibodies reacts with lymphocytes, lymphoid leukemia cells, platelets, or erythrocytes. The antigen recognized by AML-2-23 was shown to be a glycoprotein secreted by cultured human

monocytes and by HL-60 cells induced to differentiate along the monocyte lineage, thus suggesting that it may represent an as-yet-uncharacterized monokine.⁹

We recently used DNA-mediated gene transfer and cell-sorting techniques to isolate and molecularly clone the gene encoding a myeloid cell surface glycoprotein, gp150.¹¹⁻¹³ Mouse NIH-3T3 cells were cotransfected with high-molecular weight human cellular DNA and DNA from cloned feline sarcoma virus containing the *v-fms* oncogene. The *v-fms*-transformed cells that expressed gp150 were selected by fluorescence-activated cell sorting of transformed cell populations stained with a monoclonal antibody specific for the antigen. Antigen-expressing primary transformants isolated in this way typically contained approximately 1,500 kilobases (kb) of human DNA.¹⁴ Successive rounds of cotransfection and cell sorting permitted the isolation of transformants that contained limited human DNA sequences, including those that encoded gp150.

Capitalizing on these methods, we have now isolated transformed mouse cell populations that express gp55 and display concordant reactivities with each of 13 distinct monoclonal antibodies. Independently derived tertiary subclones shown to express gp55 contained a limited subset of human DNA sequences, which indicated that the epitopes recognized by our panel of antibodies were restricted to a product of a single human gene.

MATERIALS AND METHODS

Isolation of peripheral blood leukocyte fractions enriched for monocytes, lymphocytes, and granulocytes. Heparinized peripheral blood was obtained by venipuncture from normal adult volunteers with their informed consent. Mononuclear cell fractions were selected after centrifugation in Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) as described by others.^{15,16} To separate adherent from nonadherent cells, the mononuclear fraction was washed once in Hanks' balanced salt solution (HBSS), resuspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS), and incubated at 37 °C for two hours in plastic tissue culture flasks. Adherent cells were removed from the flask by scraping after incubation for five minutes at 37 °C in 3 mL of 10 µg/mL buffered

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trypsin (GIBCO, Grand Island, NY). Nonadherent cell fractions typically contained >90% lymphocytes, with the remainder consisting of monocytes as determined by microscopic examination of Cytospin slide preparations. Adherent cell fractions included 50% to 90% monocytes and contaminating lymphocytes.

Erythrocytes and granulocytes in the Ficoll-Hypaque pellet were resuspended in an equal volume of 0.4% Dextran T-500 (Sigma Chemical Co, St Louis) in HBSS and allowed to sediment for one hour on ice at unit gravity. The granulocyte-rich supernatant was withdrawn and washed twice in hypotonic lysis buffer consisting of 10 mmol/L KHCO₃ (pH 7.4), 155 mmol/L NH₄Cl, and 0.1 mmol/L EDTA to lyse residual erythrocytes. The remaining cells, typically >90% granulocytes as determined by morphological analysis of cytopsin preparations, were resuspended in RPMI 1640 medium with 5% FCS.

Transfection of high-molecular weight DNA. DNA from the cloned McDonough strain of feline sarcoma virus containing the *v-fms* gene¹⁷ was mixed with 5 µg of sheared cellular DNA (ten passages through a 25-gauge needle) and cotransfected into NIH-3T3 cells after precipitation with calcium phosphate as previously described.¹¹⁻¹³ Foci of transformed cells in multiple flasks were counted after ten days, allowed to overgrow the contact-inhibited untransformed NIH-3T3 cells, removed with trypsin, and pooled into groups for fluorescence-activated cell sorting. Control plates of NIH-3T3 cells were transfected with NIH-3T3 DNA alone and with donor DNA alone.

Monoclonal antibodies. A panel of monoclonal antibodies (Table 1) was used to detect epitopes on the surface of human

monocytes. The monoclonal antibodies MY4, B44.1, and LeuM3 were used to stain transformed recipient NIH-3T3 cells by fluorescence-activated cell sorting. Transformed cell populations selected with one of the antibodies were tested for reactivity with the entire panel. Antibodies Mol, Mo2, MY4, MY7, and MY9 were purchased from Coulter Immunology (Hialeah, FL). Antibodies LeuM3 and LeuM2 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). Antibodies 61D3 and 63D3 were purchased from Bethesda Research Laboratories (Gaithersburg, MD). All other antibodies were gifts from the investigators who derived them. In addition, the monoclonal antibodies W6/32 (Accurate Chemical and Scientific Corp, Westbury, NY) and I2 (Coulter Immunology), specific for human HLA class I and class II molecules, respectively, were included in the test panel. Mouse myeloma protein UPC10 (IgG2a; Litton Bionetics, Kensington, MD) was used as a control for nonspecific binding.

Flow cytometry. Cell cultures were washed with Ca⁺⁺-MG⁺⁺-free phosphate-buffered saline (PBS) and treated with 3 mL of 10 µg/mL buffered trypsin (GIBCO) for five minutes at 37 °C to release the adherent cells. The cells were collected, centrifuged, and resuspended in staining medium (Dulbecco's modified Eagle's medium with 5% FCS, 6 mmol/L L-glutamine, 10 mmol/L HEPES, and 2 mmol/L sodium azide). Aliquots of 50 µL containing 1 × 10⁶ cells were washed once in the same medium and incubated for 30 minutes on ice with 50 µL of medium that contained a titrated excess of monoclonal antibody. After two washes, the cells were incubated for 30 minutes on ice in 50 µL of medium containing a titrated excess of affinity-purified, fluorescein-conjugated goat antibody to mouse immunoglobulins G and M (Tago Diagnostics, Burlingame, CA). The cells were then washed twice and resuspended at 2 × 10⁶ cells/mL in medium containing 0.25 mmol/L propidium iodide.

Transfected cells were analyzed and sorted with an EPICS 753 flow cytometer (Coulter Corporation, Hialeah, FL) as previously described.¹¹ Briefly, the brightest 2% to 4% of cells were sorted, grown to confluence, and resorted until a population of cells positive for antibody binding was isolated. The brightest 10% to 15% of fluorescence-positive cells were sorted several more times to enrich for transformed cells exhibiting the highest density of antigen expression. Subpopulations of peripheral blood leukocytes were analyzed by flow cytometry following simultaneous gating on both forward-angle and orthogonal light scatter from the cells. Fluorescence profiles were obtained from monocytes within the adherent cell fraction and from lymphocytes within the nonadherent cell fraction.

Southern blot analysis. Cellular DNAs were digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III and *Sac*I. Digested DNA was subjected to electrophoresis in 0.8% agarose gels and transferred to nitrocellulose.³¹ A probe specific for human repetitive DNA sequences was prepared by self-annealing of boiled, sonicated human lymphoblastoid cell line DNA to a C₀t of 2 (where C₀ is the concentration of DNA in moles of nucleotide per liter and t is the time in seconds³²). Hybridization was performed at 60 °C at a monovalent cation concentration of 1.0 mol/L, and the double-stranded DNA was recovered by fractionation on hydroxylapatite.³² Human repetitive DNA was labeled to a specific activity of 1 × 10⁸ dpm/µg by nick translation³³ and used in blot hybridization under stringent annealing conditions.³⁴

Immunoprecipitation studies. Confluent flasks of transformed NIH-3T3 cells or adherent peripheral blood mononuclear cells were washed twice with cold PBS and incubated for 20 minutes at room temperature in 1 mL of PBS containing 5 mmol/L glucose, 3 µg lactoperoxidase, 5 mmol/L glucose oxidase (Sigma) and 1.0 mCi Na¹²⁵I (Amersham Corp, Arlington Heights, IL). Labeled cells were washed twice with PBS and lysed in 100 mmol/L Tris-HCl buffer (pH 7.6) containing 150 mmol/L NaCl, 20 mmol/L EDTA,

Table 1. Panel of Myeloid-Related Monoclonal Antibodies

Antibody	Immunoglobulin Isotype	Reported Molecular Mass (kd)	Reference
Antibodies that recognized gp55 on sorted transformants			
MY4	IgG2a	55	2
B44.1	IgM	NR	18
LeuM3	IgG2b	NR	19
63D3	IgG1	200	20
26if	IgG2b	55	6
AML-2-23	IgG2b	50-52	8
FMC17	IgG2b	NR	21
MOS1	IgG2a	NR	19
MOP39	IgG2a	NR	19
77of	IgG	55	22
23b	IgG1	55	22
49a	IgM	55	22
60bd	IgG1	55	22
Antibodies that did not recognize gp55 on sorted transformants			
Mo1	IgM	94, 155	5
Mo2	IgM	55	5
Mo4	IgM	100	23
Mo5	IgG2a	94	24
MY7	IgG1	150	2
MY9	IgG2b	67	25
121nl	IgG1	55	22
UCHM1	IgG2a	52	26
VIM13	IgM	NR	27
61D3	IgG1	75	20
5F1	IgM	85	28
20.3	IgM	NR	29
LeuM2	IgG2b	120	30

Abbreviation: NR, not reported.

1% Triton X-100, 1% deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), 2% aprotinin (Sigma) and 1 mmol/L phenylmethylsulfonyl fluoride. Immunoprecipitation was performed as previously described¹¹ with rabbit antimouse immunoglobulin and protein A-Sepharose CL-4B (Pharmacia) used as the immunoabsorbent. Gel electrophoresis was performed under either nonreducing or reducing conditions on 10% SDS-polyacrylamide gels.³⁵ The molecular weights of the precipitated bands were estimated from the mobilities of known molecular weight standards (Sigma).

RESULTS

DNA-mediated gene transfer. High-molecular weight cellular DNA from normal human peripheral blood monocytes was cotransfected into mouse NIH-3T3 cells together with a plasmid containing the molecularly cloned *v-fms* gene from the McDonough strain of feline sarcoma virus. Cells expressing the *v-fms*-transforming gene were allowed to overgrow contact-inhibited NIH-3T3 cells, typically forming 250 to 750 discrete foci of morphologically transformed cells per 100 nanograms of cloned *v-fms* DNA. The transformed NIH-3T3 cells were subjected to sequential rounds of fluorescence-activated cell sorting to select those cells that bound the monoclonal antibodies MY4, B44.1, or LeuM3. The cells with the brightest fluorescence (top 2% to 4%) were sorted and grown to confluence; this procedure was repeated three to six times to isolate rare cells expressing human monocyte antigens (Fig 1). Positive cells showed equivalent binding of each of the three monoclonal antibodies, regardless of which antibody was used to select the positive cell population. As a control, one of the original populations of transfected cells was sorted with the monoclonal antibody W6/32, which recognizes human histocompatibility class I antigens.³⁰ Five successive rounds of sorting produced a cell population positive for W6/32 binding that did not bind any of the other monoclonal antibodies tested.

DNA was extracted from primary transformants selected for binding of B44.1 and again cotransfected with *v-fms* into NIH-3T3 cells. After three successive rounds of sorting with the monoclonal antibody B44.1, fluorescence-positive secondary transformants were isolated. DNA from secondary transformants was cotransfected with *v-fms* in three independent tertiary rounds of transfection. Pooled cells from each tertiary transfection were sorted after staining with either MY4 or B44.1. Three independently derived fluorescence-positive tertiary cell populations were isolated and plated in soft agar. Subclones derived from the resulting colonies were tested with an extensive panel of monocyte-specific monoclonal antibodies (Table 1) and demonstrated positive binding with ten reagents in addition to MY4, B44.1, and LeuM3 but not with 13 others (Fig 2). Concordant binding of these 13 monoclonal antibodies by independently derived, tertiary NIH-3T3 subclones indicated that these antibodies recognize epitopes on the product of a single human gene.

Southern blot analysis of human DNA in transformed mouse cells. Cellular DNAs from a primary transformant and two independently derived tertiary subclones were analyzed for the presence of human repetitive DNA sequences. The DNAs were digested with *Bam*HI, *Eco*RI, *Hind*III, and *Sac*I and analyzed by Southern blotting. As shown in Fig 3,

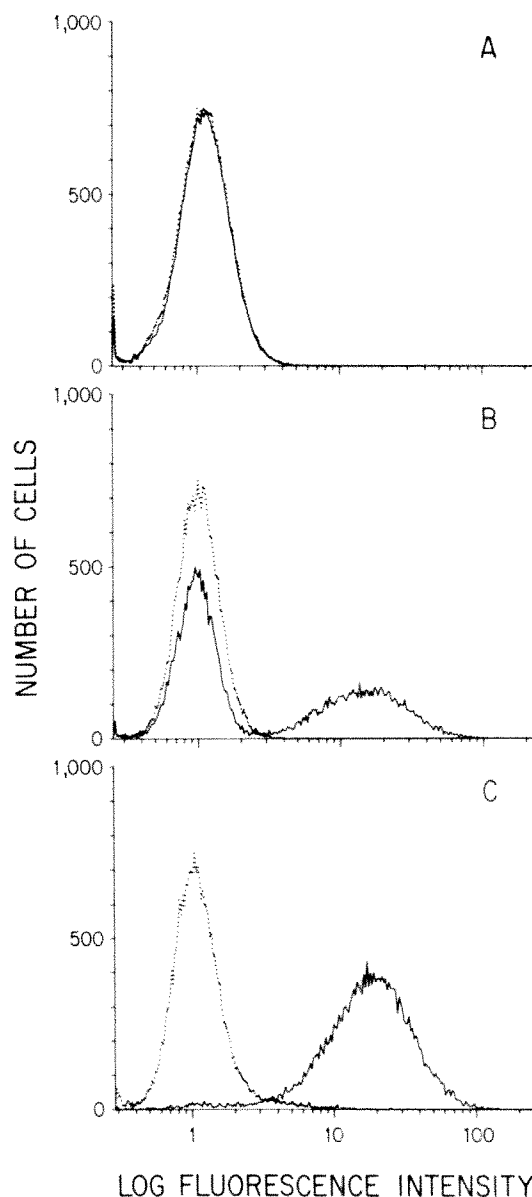


Fig 1. Selection of mouse NIH-3T3 cell transformants expressing the human monocyte membrane antigen recognized by the monoclonal antibody MY4. In each plot the fluorescence profile recorded after staining with MY4 (solid line) was compared with the fluorescence profile obtained with control mouse myeloma protein (dotted line). In panel A, pooled transformed NIH-3T3 cells were analyzed following cotransfection with human monocyte DNA; the fluorescence profile resulting from staining with MY4 was identical to the control profile. The brightest 2% of cells were selected by sterile sorting, grown up, and resorted. After two successive sorts, approximately 45% of the cells displayed high levels of MY4 fluorescence (B). After a fourth sort, >98% of the cells expressed the human monocyte cell surface antigen recognized by MY4 (C).

mouse NIH-3T3 DNA did not hybridize to the human repetitive sequence probe, whereas DNA from a primary transformant (lane 1) showed a diffuse hybridization pattern. In contrast, discrete human restriction fragments were shared by the two independently derived tertiary transfor-

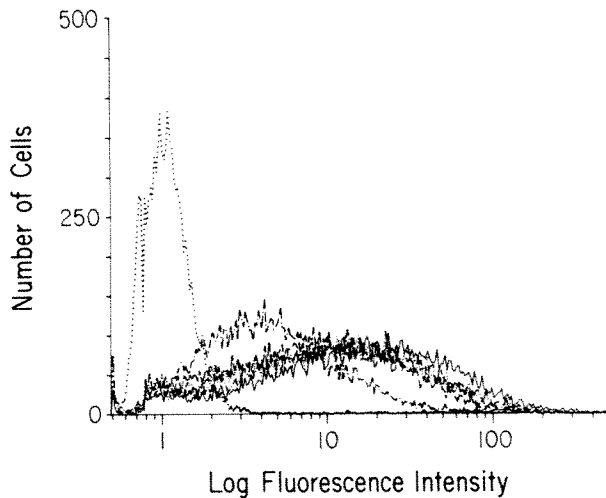


Fig 2. Analysis by flow cytometry of cell surface binding by human monocyte-specific monoclonal antibodies to a cloned tertiary NIH-3T3 transformed cell population. Positive fluorescence profiles were obtained after staining of a representative subclone with monoclonal antibodies MY4 (—), B44.1 (— · — · —), LeuM3 (— · — · —), 26IF (— · — · —), AML-2-23 (— · — · —), and 63D3 (— · — · —). Similar positive profiles were observed after staining with FMC17, MOS1, MOP39, 77of, 23b, 49a, and 60bd. Staining with all other antibodies in the panel (Table 1) resulted in negative fluorescence profiles identical to that obtained with control mouse myeloma protein (· · · · ·).

mant. Analysis of the sizes of the shared human restriction fragments indicated that the gene encoding gp55 must be equal to or less than 50 kb.

Analysis of the gp55 gene product. Figure 4 demonstrates that a representative monoclonal antibody, 26if, specifically immunoprecipitated a polypeptide common to lysates of either normal human adherent peripheral blood mononuclear cells or one of the tertiary transformants. A second, weakly labeled polypeptide with a slightly lower molecular weight was also precipitated from lysates of tertiary transformants. The electrophoretic mobility of the major polypeptide was greater on nonreducing gels (63 kd, Fig 4) than on reducing gels (55 kd, analysis not shown). NIH-3T3 cells that were transformed by *v-fms* alone did not yield any specifically immunoprecipitated products (analysis not shown).

Monoclonal antibodies that reacted with transformed mouse cells expressing gp55 were also tested for reactivity with subpopulations of normal human peripheral blood leukocytes. Although these antibodies all recognize gp55 epitopes on transfected cells and human monocytes, their binding patterns with granulocytes and myeloid precursors are reportedly different. The six antibodies in our panel that were tested showed similar patterns of reactivity (Fig 5). Each reacted with gp55 expressed at high density by monocytes but did not bind to lymphocytes. Granulocytes consistently bound gp55-specific antibodies at much lower levels than did monocytes, but the binding was specific and significantly greater than that observed with a control mouse myeloma protein.

DISCUSSION

DNA-mediated gene transfer and cell-sorting techniques were used to isolate human DNA that included the gene encoding the monocyte-specific cell surface glycoprotein gp55. Southern blot analysis detected a limited complement of human DNA sequences shared by two independently derived tertiary mouse cell transformants, thus indicating that the gene encoding gp55 comprises fewer than 50 kb of human DNA. Concordant binding with each of 13 separate monoclonal antibodies indicates that each of these antibodies binds an epitope of gp55.

The presence of bona fide gp55 on transformed mouse cells was verified by immunoprecipitation with monoclonal antibody 26if, which precipitated proteins of identical size from radiolabeled plasma membranes of normal adherent mononuclear cells or the tertiary transformants. The presence of an additional, less efficiently labeled band of slightly lower molecular weight in lysates of monocytes and tertiary transformants suggests that a small fraction of cell surface gp55 molecules may have altered or incomplete posttranslational modification, the exact nature of which is unknown. The electrophoretic mobility of the major precipitated polypeptide on reducing gels (55 kd) agrees with published reports of a 55-kd glycoprotein immunoprecipitated by My4,⁴ a 50- to 66-kd glycoprotein by AML-2-23,⁹ and a 55-kd glycoprotein by 26if,⁶ but conflicts with accounts of a 200-kd protein precipitated by 63D3.²⁰ In our experiments, the antibodies LeuM3, 63D3 and FCM17 failed to yield specific immunoprecipitable products.

The binding specificities observed in this study are also strikingly similar to those reported for Mo2, which specifically immunoprecipitates a 55-kd glycoprotein from monocytes under reducing conditions.⁵⁻⁷ Additionally, Todd²² has shown that antibodies 26if, 77of, 60bd, and 49a, 23b, and 121nl all competitively block binding of directly fluorescently labeled Mo2 to monocytes. The lack of Mo2 binding to our tertiary transformants suggests that Mo2 might recognize an epitope that results from a posttranslational modification of monocyte gp55, which is altered in mouse cell transformants. Alternatively, Mo2 and 121nl might react with the product of another gene that assembles noncovalently with gp55 at the cell surface.

Reported experience with the 13 monoclonal antibodies that detected gp55 on our transformed cells indicates that all of the reagents bind to monocytes, with variable binding to granulocytes and none to lymphocytes. Accordingly, six gp55-specific antibodies that we tested (MY4, LeuM3, 63D3, 26if, B44.1, and AML-2-23) displayed strong specific affinity for peripheral blood monocytes as well as background levels of binding to lymphocytes and uniform but weak binding to granulocytes. These antibodies were previously reported to stain myeloid leukemia cells with monocytic morphology, consistent with their specificity for monocytes in populations of circulating leukocytes.¹⁰

The expression of gp55 by mature monocytes and macrophages suggests the possibility that it may mediate one of the diverse functional roles of these cells. Sorted 63D3-positive or 26if-positive cells from peripheral blood have been asso-

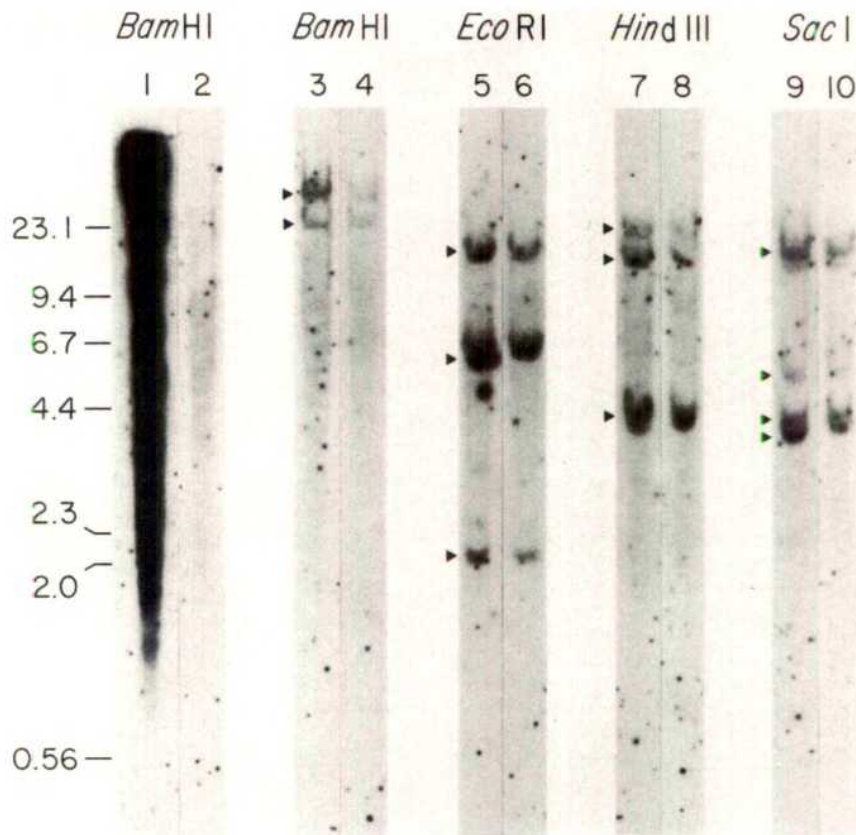


Fig 3. Southern blot analysis of human DNA sequences in transformed mouse cells. DNA samples were digested with the indicated enzymes before electrophoresis and transfer to nitrocellulose. Hybridization was performed by the use of radiolabeled human DNA consisting of highly reiterated sequence families. The lanes shown were composed from the same blot. DNA samples were obtained from a primary transformant (lane 1), control NIH-3T3 cells (lane 2), and two independently derived tertiary subclones (lane 3, 5, 7, 9 and lanes 4, 6, 8, 10, respectively). Major restriction fragments present in both tertiary subclones are indicated by triangles. The positions of bacteriophage lambda *Hind*III fragments of known length (noted in kilobase pairs) are indicated to the left of the figure.

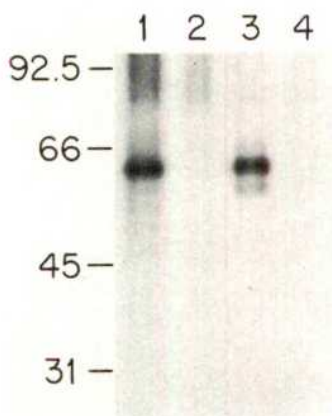


Fig 4. Immunoprecipitation of gp55 from normal human monocytes and from mouse NIH-3T3 tertiary transformants. Cells were enzymatically surface radioiodinated with ^{125}I , detergent lysates were precipitated with antibody, and the radiolabeled immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under nonreducing conditions. Lysates from normal human monocytes (lanes 1 and 2) or a tertiary NIH-3T3 subclone (lanes 3 and 4) were precipitated with monoclonal antibody 26if (lanes 1 and 3) or an isotype-matched control mouse myeloma protein (lanes 2 and 4). Positions of molecular weight standards are indicated.

ciated with accessory cell function and antigen presentation, respectively.^{36,37,7} Maliszewski et al⁹ concluded that gp55 is secreted by cultured monocytes and 1,25-dihydroxyvitamin D₃-treated HL60 cells that display monocytoid differentiation and is present in a slightly lower molecular weight form (44 to 50 kD) in normal serum.⁹ Thus, gp55 may be a polypeptide hormone precursor for a monokine of an as-yet-undetermined function. The transfer and expression of sequences encoding gp55 in a mouse genetic background should facilitate the characterization of this gene and its regulation and expression by normal and malignant cells.

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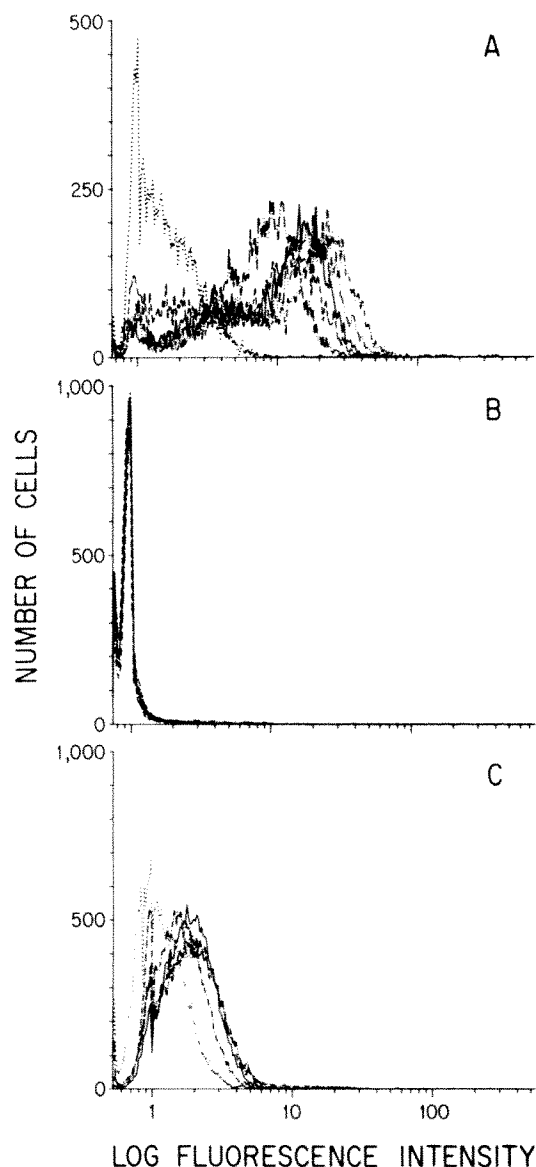


Fig 5. Flow cytometric analysis of cell surface binding of six human monocyte-specific antibodies that recognize epitopes of gp55. The fluorescence profiles of human peripheral blood cells are displayed after staining of the cells with monocyte-specific monoclonal antibodies MY4 (—), LeuM3 (—), B44.1 (—), AML-2-23 (—), 63D3 (—), or 26if (—). Fluorescence profiles are shown after staining of normal human peripheral blood monocytes (A), lymphocytes (B), or granulocytes (C). In each panel, the fluorescence profiles are compared with that obtained with control mouse myeloma protein (.....).

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Chronic Myelogenous Leukemia: Amplification of a Rearranged *c-abl* Oncogene in Both Chronic Phase and Blast Crisis

By Steven J. Collins and Mark T. Groudine

The specific genetic events that distinguish the blast crisis from the chronic phase cells of chronic myelogenous leukemia (CML) are unknown. The most common karyotypic change that occurs as CML evolves from chronic phase to blast crisis is the development of multiple Philadelphia (Ph¹) chromosomes, each of which is presumably harboring a translocated *c-abl* oncogene. We describe here a patient with CML who presented in lymphoid blast crisis with three Ph¹ chromosomes/metaphase associated with an amplified, rearranged *c-abl* oncogene fragment and high levels of the aberrant 8-kilobase *bcr-abl* transcript. This

rearranged *c-abl* fragment was amplified to a similar degree in both the patient's blast crisis cells and in his terminally differentiated granulocytes, but the level of the aberrant CML-specific *bcr-abl* transcript was some eight- to 16-fold higher in the blast crisis cells v the granulocytes. This analysis indicates that genomic amplification of a translocated *c-abl* oncogene, although perhaps important in the evolution of CML, nevertheless cannot, by itself, be the sole genetic event giving rise to blast crisis.

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CHRONIC MYELOGENOUS LEUKEMIA (CML) is a clonal disorder involving a pluripotent stem cell from which granulocytes, erythrocytes, platelets, monocytes, macrophages, and B lymphocytes arise.¹ Over 90% of cases of CML exhibit the Philadelphia chromosome (Ph¹)(22q-), which results from a reciprocal translocation involving the distal portions of the q arms of chromosomes 9 and 22 and which is the most specific chromosome abnormality associated with any human malignancy. A prominent feature of CML is the distinct clinical phases of the illness. The relatively benign chronic phase, characterized by hyperplasia of terminally differentiated granulocytes, inevitably progresses to the blast crisis phase in which immature blasts predominate and that usually proves lethal.

Recent observations have provided considerable insight into the molecular genetics of the Ph¹ chromosome formation in CML. The *c-abl* oncogene is defined by virtue of its homology to *v-abl*, the transforming sequences of the Abelson murine leukemia virus.² In CML, this oncogene is translocated from its usual position on the distal end of the q arm of chromosome 9 to a relatively restricted region on the Ph¹ termed the breakpoint cluster region (*bcr*).^{3,4} As a result of this translocation, a hybrid *bcr-abl* transcript is generated.^{5,6} This abnormally large transcript (8.2 kilobase [kb]) probably codes for the abnormally large *abl*-related protein product (P210) that has been demonstrated in CML cells.^{7,8} Although the physiological function of the *c-abl* oncogene is unknown, these findings indicate that abnormalities in the structure and expression of this oncogene may be critical to the pathogenesis of CML.

Several lines of evidence indicate that enhanced expression of the altered *c-abl* oncogene may be important in the progression of CML from chronic phase to blast crisis. We have previously demonstrated that this oncogene is amplified some four- to eightfold in the K-562 CML blast crisis cell line.⁹ The development of multiple Ph¹ chromosomes, each of which is presumably harboring a translocated *c-abl* oncogene, is the most frequent karyotypic abnormality that occurs as CML evolves from the chronic phase to blast crisis.¹⁰ In addition, enhanced expression of the abnormal 8.2-kb *bcr-abl* hybrid transcript is noted in CML blast crisis cells v chronic phase cells.¹¹ Thus, one genetic event leading to enhanced expression of the translocated *c-abl* oncogene during progression of CML from the chronic phase to blast

crisis may be an increase in the copy number of the abnormal *bcr-abl* locus.

Is the enhanced expression of the aberrant 8.2-kb *bcr-abl* transcript due solely to the presence of the multiple Ph¹ chromosomes in certain CML cells? Is the presence of multiple Ph¹ chromosomes the main genetic change distinguishing CML chronic phase from blast crisis? We have been able to approach these questions by using a molecular genetic analysis of blast crisis and chronic phase cells from a patient with CML who presented in lymphoid blast crisis with multiple Ph¹ chromosomes/metaphase associated with amplification of a rearranged *c-abl* oncogene. Although there was a marked difference in the level of expression of the aberrant *bcr-abl* transcript in the blast crisis v the chronic phase cells, both of these cell types in this patient exhibited similar amplification of the rearranged *c-abl* fragment, which suggests that both the blast crisis and chronic phase cells harbored multiple Ph¹ chromosomes.

MATERIALS AND METHODS

Case Summary

The patient was a 42-year-old man who presented with a 1-month history of weight loss, malaise, fatigue, and headaches. His physical exam revealed pallor and splenomegaly without adenopathy. A complete blood cell count (CBC) revealed a hematocrit value (Hct) of 29, platelet count of 32,000, and a WBC count of 430,000 with

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54% blasts, 3% lymphocytes, 4% monocytes, 3% nucleated RBCs, 10% myelocytes, 3% metamyelocytes (metas), 7% bands, and 16% segmented neutrophils (segs). A bone marrow aspirate and biopsy sample revealed a marrow packed with over 90% blasts that were terminal transferase-positive. Karyotypic examination revealed 52 XXY, +5, +11, +20, +3 Ph¹ in virtually all unstimulated metaphases. In contrast, phytohemagglutinin (PHA)-stimulated metaphases displayed a 46 XY karyotype. Therapy was initiated with oral hydroxyurea followed by two 3-week courses of vincristine, prednisone, and Adriamycin. Six weeks following initiation of therapy, the patient was noted to be in partial remission with an Hct of 28, platelet count of 56,000, and a WBC count of 7,000 with 3% blasts, 8% lymphocytes, 8% monocytes, 5% nucleated RBCs, 11% myelocytes, 12% metas, 15% bands, and 38% segs. The patient refused further chemotherapy and was discharged. Two months later he returned with a peripheral WBC count of 110,000 consisting of greater than 90% lymphoblasts. Therapy with oral hydroxyurea was reinstituted.

Cell Separation

At initial presentation the peripheral blood was diluted 1:1 with plain RPMI, layered over isolymph (Gallard/Schlesinger, Carle Place, NY), and spun at 1,800 rpm for 20 minutes. The interface cells were washed several times in RPMI and upon Wright staining were found to consist of over 85% blasts, with the remaining cells being predominantly myelocytes and metamyelocytes. The RBC-granulocyte isolymph pellet was brought to 25 mL with plain RPMI and mixed with 25 mL 3% dextran (T-500) in phosphate-buffered saline. The RBCs were allowed to sediment for 1 to 1.5 hours, and the supernate was harvested and the cells pelleted. The contaminating RBCs were lysed several times in 0.2% NaCl. The pelleted cells were then washed several times in plain RPMI and consisted of greater than 95% mature granulocytes.

After the chemotherapy-induced partial remission, peripheral blood was subjected to 3% dextran sedimentation followed by lysis of RBCs as described above. These cells consisted of approximately 70% metamyelocytes, bands, and segs, with the remaining cells consisting of mature lymphocytes and monocytes. There were fewer than 5% blasts in this cell population.

At the time of relapse, mononuclear cells were isolated following isolymph gradient centrifugation of peripheral blood. This fraction consisted of greater than 95% blasts.

DNA and RNA Extractions

DNA extractions from the various leukocyte populations were performed by digesting nuclei with protease K followed by phenol/chloroform extraction and ethanol precipitation as previously described.¹¹ RNA was extracted by homogenizing the cells in guanidine thiocyanate followed by ultracentrifugation through a cesium chloride cushion as described by Chirgwin et al.¹² Poly A⁺ RNA was selected by using oligo-dT cellulose.¹³

Southern, Northern, and Dot Blot Hybridizations

Restriction endonuclease digestion of genomic DNA was performed following manufacturer specifications. Southern and Northern blots were made following standard procedures.¹⁴ RNA dot blot analysis on the total cellular RNA was performed as previously described.¹¹ All blots were hybridized to nick-translated probes and subsequently washed under exactly the same conditions described previously.¹¹

Molecular Probes

5' abl. A molecular probe from the 5' end of the *c-abl* oncogene was cloned from a genomic library constructed from the K-562

CML blast crisis cell line.¹⁵ DNA from this cell line was partially digested with *Sau* 3A and fractionated by sucrose density gradient centrifugation, and the 15- to 23-kb DNA fragments were isolated. This DNA was ligated with *Bam*-digested EMBL 3 phage vector DNA, and the ligation mixture was packaged in vitro. Approximately 500,000 recombinant clones were screened with a nick-translated 550-bp *Hinc*II-*Sma*I fragment derived from the pAB1 sub9 plasmid, which contains a *v-abl* insert.¹⁶ This 550-bp fragment represents the most 5' sequences of *v-abl*. Following screening, ten positive clones were isolated and purified. One of these clones contained a 17-kb genomic insert that exhibited a restriction map similar to that of the previously published 5' end of *c-abl*,¹⁷ and from this clone a 0.3-kb *Hind*III-*Eco*R_I fragment free of human repetitive sequences was isolated and used as a molecular probe.

Genomic bcr probe. A 1.4-kb *Bgl*II-*Sst* human genomic fragment representing sequences within the *bcr* gene that are immediately 5' to the *bcr* itself¹⁸ was isolated from a normal genomic library. Details of the cloning procedure will be published elsewhere.

bcr cDNA probes. A 2-kb *bcr* cDNA clone was isolated from an HL-60 cDNA library screened with the aforementioned 1.4-kb *Bgl*II-*Sst* human genomic *bcr* fragment. A 700-bp *Pst* fragment near the 3' end of this clone that contains sequences that are represented in the normal *bcr* transcript but not in the aberrant *bcr-abl* transcript^{5,6,18} was utilized as a probe designated 3' *bcr*.

J_H. A 3.2-kb R_I-*Hind*III genomic probe cloned into pBR 322, which encompasses several of the exons within the J_H region of the immunoglobulin heavy chain locus, was obtained from Dr Roger Perlmutter.

v-abl. This probe is a 1.5-kb *Bgl*II fragment isolated from the *v-abl* insert of the pAB1 sub9 plasmid.¹⁶

Actin. The actin probe used is a 2.0-kb chick pA 1 β actin cDNA *Pst* fragment.¹⁹

In all cases the probes were purified from their respective vectors using the glass bead technique of Vogelstein and Gillespie²⁰ prior to nick translation and hybridization to the appropriate blots.

RESULTS

Cell Harvesting and Separation

We obtained peripheral blood cells from this patient at three different times during the course of his illness including (1) at initial presentation, at which time he had a WBC count of 430,000 with 54% blasts; (2) during a brief chemotherapy-induced partial "remission" when his peripheral count was 7,000 with >70% terminally differentiating granulocytes and <5% blasts; and (3) during relapse of blast crisis when his peripheral count rose to greater than 100,000 with over 90% blasts. At initial presentation, we separated the immature blast cells from the mature terminally differentiated granulocytes by isolymph gradient centrifugation as described in Materials and Methods. DNA and RNA were extracted from these cells as well as from the mature granulocytes isolated at the time of partial remission and from the immature blasts isolated at the time of relapse.

Lymphoid Nature of the Blast Crisis Cells

Many cases of CML blast crisis are B lymphoid in origin and exhibit immunoglobulin gene rearrangements.²¹ This patient's blast cells were positive for terminal transferase, thus suggesting their lymphoid origin. This was confirmed by Southern blot analysis of the blast cell DNA, which revealed several extra nongermline bands hybridizing to a heavy-chain immunoglobulin joining region probe (J_H) (Fig 1). The

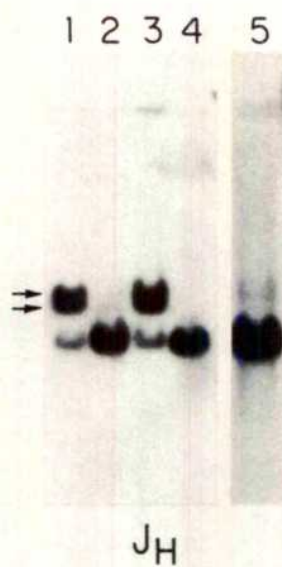


Fig 1. Rearrangement of the immunoglobulin heavy-chain locus in the CML blasts. Genomic DNA was digested with *Bgl*II, electrophoresed in 1.0% agarose, transferred to nitrocellulose, and hybridized to a ^{32} P-labeled J_H probe specific for the immunoglobulin heavy-chain joining region. Samples include CML blasts isolated at presentation (lane 1); initial presentation granulocytes (lane 2); blasts isolated at relapse (lane 3); HL-60 cells, which exhibit the germline pattern (lane 4); and partial remission CML cells (lane 5). Rearranged fragments in the CML blasts are indicated by arrows.

identical restriction pattern was seen with this probe in DNA from both the blasts at initial presentation and at relapse (Fig 1). In contrast, predominantly the germline pattern was seen in the granulocyte populations isolated at initial presentation and during the chemotherapy-induced partial remission (Fig 1). Therefore these blots not only reveal the B lymphoid nature of the patient's blasts but also indicate that the separated granulocyte fraction is not contaminated with any significant number of these blasts.

RNA Analysis

RNA from the patient's blast and initial presentation granulocyte populations were subjected to Northern and dot blot analysis. The patient's blast cells exhibited the abnormal 8.2-kb *bcr-abl* hybrid transcript that has been previously described in Ph^1 chromosome-positive CML cells^{5,6,11} (Fig 2). The relative amount of this transcript is quite high and is comparable with that found in the K-562 cell line, which exhibits a four- to eightfold amplification of the *c-abl* oncogene (Fig 2). Although we did not obtain enough granulocyte RNA to perform Northern blots, we did note by RNA dot blot analysis a marked decrease of at least eightfold in the level of *abl*-related transcripts in the granulocyte population compared with the blasts (Fig 3). In addition, a similar eightfold decrease in the level of *bcr*-related transcripts was noted in the granulocyte population v the blasts. These results indicate that the level of *bcr-abl* hybrid transcripts is significantly higher in the blasts v the granulocyte

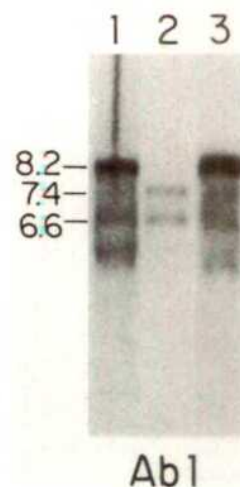


Fig 2. Enhanced expression of the aberrant 8.2-kb *bcr-abl* transcript in the CML blasts. Poly A⁺ RNA (5 μ g/line) was electrophoresed in a formaldehyde gel, transferred to nitrocellulose, and hybridized to a *v-abl* probe. The normal 7.4- and 6.6-kb transcripts as well as the aberrant 8.2-kb transcripts are noted. RNA samples are from K-562 (lane 1), HL-60 cells (lane 2), and the patient CML blasts (lane 3).

population in this patient. It is of interest that hybridization of this dot blot to a 3' *bcr* probe, which measures levels of *bcr* transcripts from normal chromosome 22 but does not detect *bcr-abl* hybrid transcripts, reveals a similar decrease in levels of normal *bcr* transcripts in the granulocyte populations v the blasts (Fig 3). As a control, we hybridized these same samples to an actin probe that revealed similar levels of actin-related transcripts in both (Fig 3).

c-abl Rearrangement and Amplification in Both Blasts and Granulocytes

We hybridized Southern blots of restriction digests of DNA from patient blasts and initial presentation granulocyte fractions with a 300-bp R_1 -*Hind*III *c-abl* probe (probe

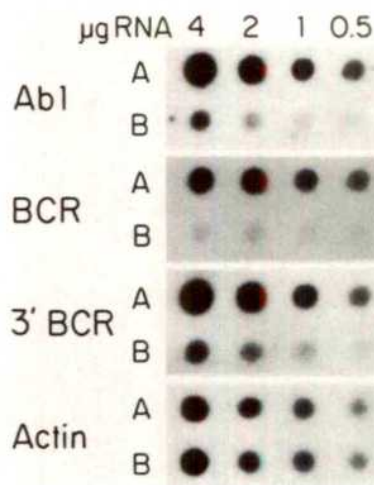


Fig 3. Enhanced expression of both *abl*- and *bcr*-related transcripts in the CML blasts (row A) v initial presentation CML granulocytes (row B). The indicated quantities of RNA were dotted onto nitrocellulose and hybridized sequentially to the indicated probes. Between successive hybridizations, probes were removed from the blot by the addition of boiling water followed by cooling to room temperature with constant rocking. Exposure time was 20 hours for the *v-abl*, *bcr*, and 3' *bcr* probes and 4 hours for the actin probe. The 2-kb *bcr* cDNA probe recognizes both normal *bcr* transcripts as well as *bcr-abl* fusion transcripts. The 700-bp 3' *bcr* probe recognizes only normal *bcr* transcripts.

B) located within a 12-kb *Bgl*II fragment on chromosome 9 at the 5' end of *c-abl* (Fig 4). This probe detected an extra fragment in both the blast and granulocyte DNA digests, thus indicating that the break on chromosome 9 in this patient lies within this 12-kb *Bgl*II fragment and that both the blast and granulocyte subpopulations harbor a similar chromosome 9 breakpoint (Fig 4). In both the blast and

granulocyte fractions, this probe hybridizes more intensely to the rearranged *Bgl*II fragment than to the germline fragment, which suggests that the rearranged *c-abl* fragment is amplified in this patient. We also noted a rearranged and amplified *Kpn*I fragment in genomic digests of blast and granulocyte DNA hybridized to a chromosome 22 probe (probe A) located just 5' to the breakpoint cluster region on

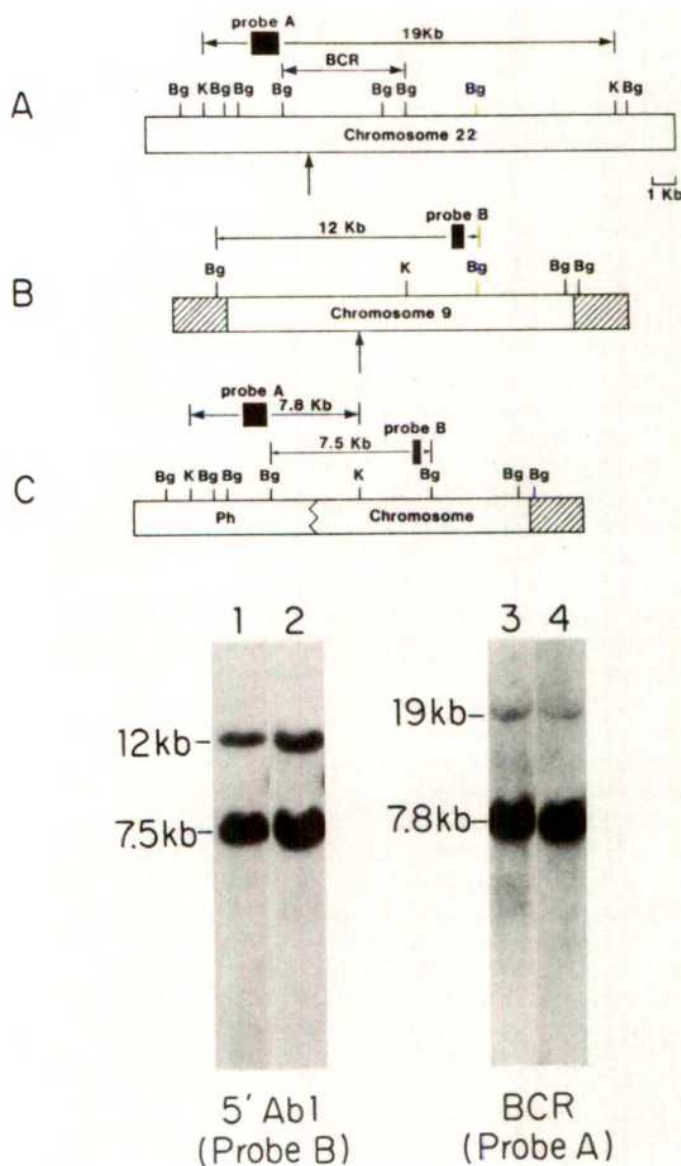


Fig 4. Amplification and rearrangement of *abl* and *bcr* sequences in CML blasts and granulocytes. Genetic maps of the *bcr* region on chromosome 22 (A), the 5' *c-abl* region on chromosome 9 (B) and the 9:22 Ph¹ breakpoint region on the Ph¹ chromosome in this particular patient (C) are shown. These maps were derived from data from references 4, 5, and 17 and from our own mapping data. The approximate breakpoints on chromosomes 22 and 9 in this patient have been previously mapped and are indicated by arrows. Probe A is a 1.4-kb *Bgl*II-*Sst* chromosome 22 fragment located immediately 5' to the breakpoint cluster region (BCR) and hybridizes to a 19-kb *Kpn*I germline fragment as indicated in A. Probe B is a 0.3-kb R₁-*Hind*III fragment located in the 5' *c-abl* region of chromosome 22 that hybridizes to a 12-kb *Bgl*II germline fragment as indicated in B. *c-abl* exons are denoted by ▨. These probes were hybridized to Southern blots of genomic digests of CML DNA from patient blasts (lanes 1 and 3) and from initial presentation patient granulocytes (lanes 2 and 4). Lanes 1 and 2 are *Bgl*II digests; lanes 3 and 4 are *Kpn*I digests. In both blasts and granulocytes probe B identifies the 12-kb germline *Bgl*II fragment as well as a rearranged, amplified 7.5-kb fragment as indicated (lanes 1 and 2). Probe A identifies the 19-kb *Kpn*I germline fragment as well as a rearranged, amplified 7.8-kb fragment as indicated (lanes 3 and 4). These rearranged fragments are 9:22 junction fragments as indicated in C. Densitometric scanning reveals a ratio of rearranged:germline fragment of approximately 6:1 using probe B (lanes 1 and 2) and 10:1 using probe A (lanes 3 and 4) in both the blast and granulocyte DNA digests.

chromosome 22 (Fig 4). Thus, both *bcr* (chromosome 22) and *c-abl* (chromosome 9) sequences are amplified and rearranged in this patient and this amplification/rearrangement most likely reflects the multiple Ph¹ chromosomes/metaphase that were demonstrated in this patient's leukemic cells. Notably, with both these probes the ratio of the intensity of hybridization of the rearranged to the germline fragments is essentially the same when comparing the blast and granulocyte DNA digests, thus indicating that the rearranged *c-abl* gene is amplified to a similar degree in both (Fig 4).

DISCUSSION

The recent identification of specific genes that are involved in Ph¹ chromosome formation in CML now allows the identification of cells harboring this marker chromosome by Southern blot hybridization of restriction digests of genomic DNA.^{3,4} The major advantage of this technique is that metaphases from immature, proliferating cells are no longer essential for analysis, since the assay can be performed with any cellular DNA at any particular level of cell differentiation. We utilized this approach to analyze various cell populations of a patient who presented with CML in blast crisis. Several somewhat unusual aspects of this patient's illness made the analysis particularly useful. At presentation there were a significant number of terminally differentiating granulocytes in his peripheral blood in addition to the lymphoid blast crisis cells, which allowed for ready separation of distinct CML cell subpopulations. Moreover, Southern blot analysis readily distinguished these lymphoid blast crisis cells from the terminally differentiating granulocytes, since only the blasts exhibited a rearrangement of the immunoglobulin heavy-chain J_H locus. In addition, a karyotype analysis of bone marrow blasts in this patient revealed three Ph¹ chromosomes/metaphase. The breakpoint on chromosome 9 that contributed to the Ph¹ formation in the leukemic cells was relatively close to the *v-abl* homologous region such that Southern blots revealed a rearranged *c-abl* fragment utilizing a 5' *c-abl* probe. (Only 15% to 20% of CML patients exhibit a breakpoint this close, with most CML chromosome 9 breakpoints occurring much further 5' to the *v-abl* homologous region.)^{4,22} This rearranged 5' *c-abl* fragment was amplified in this patient's blast cells, most likely reflecting the presence of the multiple Ph¹ chromosomes/metaphase. Thus using this 5' *c-abl* probe, we were not only able to determine the presence of the Ph¹ in various cell populations but could also, by comparing the intensity of hybridization of the amplified, rearranged fragment to the germline fragment, estimate the number of Ph¹ chromosomes that each isolated cell population harbors.

We isolated blast crisis cells from this patient at the time of initial presentation and at the time of relapse. We also isolated terminally differentiated granulocytes at initial presentation and during a chemotherapy-induced partial "remission" when the peripheral blood blast cell population fell below 5%. The morphologically immature blasts displayed a rearrangement of the immunoglobulin heavy-chain locus that indicated a B lymphoid blast crisis, whereas the mature granulocytes exhibited a germline immunoglobulin heavy-

chain pattern and were therefore representative of chronic phase cells. If one of the major genetic events that distinguishes these lymphoid blast crisis cells from the terminally differentiated chronic phase granulocytes is the development of multiple Ph¹ chromosomes with concomitant amplification of the rearranged *c-abl* fragment, then one would predict that the terminally differentiated granulocytes would not display amplification of the rearranged *c-abl* fragment (ie, precursors of these granulocytes display a single Ph¹). However, our Southern blots show that this is clearly not the case, since this rearranged *c-abl* fragment is amplified to a similar degree in both the blast and granulocyte cell populations. Indeed, these Southern blots indicate that the terminally differentiated granulocytes also harbor multiple Ph¹ chromosomes. Therefore, the development of multiple Ph¹ chromosomes per se cannot be the sole genetic event that leads to the blast crisis phenotype, and one would predict that there are other genetic factors besides multiple Ph¹ chromosomes that distinguish the blast crisis from the chronic phase cells.

One potential problem in interpreting our Southern blots is that the enhanced hybridization of the *bcr* and *abl* probes to the rearranged fragments may be secondary to preferential transfer from the agarose gel to nitrocellulose of the smaller, rearranged fragments compared with the larger, germline fragments. Indeed since the rearranged *bcr* and *abl* fragments most likely are present on each individual Ph¹ chromosome, one would predict a ratio of intensity of hybridization of rearranged:germline fragment of 3:1 with these probes (ie, three Ph¹ chromosomes/one normal chromosome 9 or 22/metaphase). However, our densitometric scanning indicates a hybridization intensity ratio of 6:1 with the 5' *c-abl* probe and 10:1 with the *bcr* genomic probe (Fig 4), which suggests that there indeed has been preferential transfer of the smaller rearranged fragments in these blots. However, the important observation is that the hybridization intensity ratios of rearranged:germline fragments using both *abl* and *bcr* probes are virtually identical in the blasts (which by karyotypic analysis harbor three Ph¹ chromosomes/metaphase) and the chronic phase granulocytes. Thus even though preferential transfer of the smaller rearranged fragments is probably present in these blots, this does not interfere with our conclusion that the chronic phase granulocytes are similar to the blast crisis cells in harboring multiple Ph¹ chromosomes.

Although we noted amplified, rearranged *c-abl* sequences in both the lymphoid blast crisis cells and the chronic phase granulocytes in this patient, the blast crisis cells had an eight- to 16-fold greater level of *bcr-abl*-related transcripts than the chronic phase granulocytes (Fig 3). Thus, the presence of amplified *c-abl* sequences per se within the genome of certain CML cells does not necessarily lead to enhanced expression of these sequences. What controls the expression of the aberrant *bcr-abl* transcript in CML? The *c-abl* oncogene in CML is translocated into the *bcr* gene on the Ph¹, and transcription of this oncogene may then come under control of those factors that normally control transcription of the intact *bcr* gene on the normal chromosome 22. Consistent with this hypothesis is our finding that there is a decrease in the normal chromosome 22 *bcr* transcripts in the CML granulocytes v blast crisis cells that is almost

exactly proportional to the decrease in the level of Ph¹ chromosome *bcr-abl*-related transcripts in these cells (Fig 3). Indeed, one hypothesis to explain the progression of CML to blast crisis is that a subset of Ph¹-positive CML cells harboring trans-activating factors specific for the normal *bcr* gene might also develop enhanced expression of the aberrant *bcr-abl* transcript and presumably gain a selective growth advantage over other Ph¹-positive CML cells that do not harbor such specific factors. This model would explain how some CML cell populations (for instance, the granulocytes from this patient) would not display high levels of the *bcr-abl* transcript even though they harbor multiple Ph¹ chromo-

somes. An alternative hypothesis to explain the evolution of CML is that a further genetic event or mutation, perhaps involving another oncogene in addition to the Ph¹ translocation, gives rise to blast crisis.

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Augmentation of In Vitro Human Marrow Erythropoiesis Under Physiological Oxygen Tensions Is Mediated by Monocytes and T Lymphocytes

By Rukmani Pennathur-Das and Lee Levitt

Tissue and marrow (BM) in vivo O_2 tensions have been estimated at 23 to 40 mm Hg (3% to 5% O_2). We have investigated cellular regulation of burst-forming units-erythroid (BFU-E) under 5% O_2 . BFU-E from BM mononuclear cells (MNC) were cultured in methylcellulose medium and erythropoietin (Ep) $\pm$ monocyte-conditioned medium (MoCM, a source of burst-promoting activity, BPA) in the presence or absence of autologous T cells (T) and/or monocytes (M ϕ) under either 5% or 21% O_2 after depletion of T (MNC-T), M ϕ (nonadherent buoyant cells, NAB) or both T and M ϕ depletion (NAB-T). MNC BFU-E growth under 5% O_2 was augmented over 0.1 to 1.5 U/mL of Ep. BFU-E augmentation under 5% O_2 was abolished by depletion of BM M ϕ , T, or both from MNC. The addition of MoCM affected neither a BFU-E increase under 5% O_2 nor the abrogation of that increase upon T or M ϕ depletion. The addition of 5% to 20% M ϕ or 10% to 20% T to NAB-T failed to restore the BFU-E increase under 5% O_2 . However, BFU-E augmentation under 5% O_2 was reestablished when 10% autologous M ϕ , 10% T, or 10% T plus 10%

M ϕ were added back to marrow NAB, MNC-T, or NAB-T. BM BFU-E was not augmented in the presence of varying concentrations of catalase, superoxide dismutase, or reduced glutathione at 21% O_2 ; moreover, BFU-E augmentation was maintained at 5% O_2 relative to 21% O_2 in the presence of each of these antioxidants. CM prepared under 5% or 21% O_2 from BM M ϕ , T, or M ϕ plus T were assessed for BPA against BM NAB-T using a sensitive BPA assay incorporating delayed Ep addition to cultures. Only CM from mixtures of M ϕ and T cells at 5% O_2 demonstrated potent BPA; little or no BPA was detected with T or M ϕ CM at 5% O_2 and at 21% O_2 or T and M ϕ CM at 21% O_2 . The sensitivity of NAB-T BFU-E to exogenous BPA was virtually identical at 21% and 5% O_2 . These results indicate that human BM BFU-E are augmented under 5% O_2 and that T cells and M ϕ together mediate that augmentation by collaborating to produce BPA-like activity in response to physiological O_2 tensions.

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IN VITRO INVESTIGATIONS of hematopoiesis have to date been performed at atmospheric oxygen tensions (140 to 150 mm Hg, 21% O_2). However, tissue oxygen tensions and, in particular, bone marrow oxygen tension are far below 140 mm Hg, the partial pressure of oxygen at room air. Guyton estimates the tissue oxygen tension in vivo to be approximately 40 mm Hg.¹ Tondevdol et al have measured medullary cavity oxygen pressures within canine long bones and have reported values between 23.5 and 27.7 mm Hg (3% to 4% O_2) at arterial pressures of 75 to 110 mm Hg. When the arterial pressure dropped below 75 mm Hg, the bone medullary oxygen tension dropped still further to 14 to 18 mm Hg.^{2,3} Thus, the true physiological pO_2 in the marrow is much closer to 5% O_2 than 21% O_2 , and in vivo hypoxic conditions would be expected to cause further decrements in marrow oxygen tension.

Although it has been known for a number of years that compensatory in vivo erythropoietic responses to hypoxia are induced by increased erythropoietin (Ep) production⁴ and that tissue oxygen delivery can be augmented through increased production of 2,3-diphosphoglyceric acid,⁵ in vitro regulation of erythropoiesis under less than atmospheric oxygen tensions has not been investigated in detail. Bradley et al have reported an increased size and number of hematopoietic granulocyte-macrophage progenitor cells (CFU-GM) when murine marrow is cultured in agar and incubated at 6.8% O_2 .⁶ Rich and Kubanek have reported increased growth of both early (BFU-E) and late (CFU-E) erythroid progenitors when murine marrow cells were cultured at 5% O_2 .⁷ They attributed this increased erythroid-plating efficiency to lowered oxygen toxicity at 5% O_2 and observed that antioxidants augmented erythroid colony formation when marrow cells were cultured under atmospheric oxygen tensions. However, antioxidants did not reproduce the degree of erythroid augmentation observed at 5% O_2 . Rich and Kubanek speculated that either a further reduction in oxygen toxicity or recruitment of additional erythroid colony-form-

ing cells may occur when murine marrow is cultured at physiological oxygen tensions. Broxmeyer in a preliminary report has recently observed increased human granulocyte-macrophage progenitor (CFU-GM) formation at 5% O_2 and has suggested that CFU-GM progenitors demonstrate increased sensitivity to granulocyte-macrophage colony-stimulating factors (GM-CSF) when grown at 5% O_2 .⁸ He also observed an increased release of GM-CSF from human peripheral blood macrophages cultured at low O_2 tensions. Investigators have also recently reported that human marrow erythropoiesis is augmented under low pO_2 tensions, but the mechanism of that augmentation has not been well delineated.^{9,10}

In this study, we have investigated the effects of physiological (5%) O_2 tensions on human marrow erythropoiesis and have evaluated specific cellular contributions to erythroid progenitor growth under physiological oxygen tensions. Our

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results indicate that erythroid colony formation is enhanced when human marrow is cultured at 5% O₂ and that the erythropoietic augmentation observed at physiological oxygen tensions is mediated by a cooperative interaction between monocytes (M ϕ) and T cells.

MATERIALS AND METHODS

Bone marrow procurement and cell separation methods. All studies were approved by the Stanford University Human Experimentation Committee. All patients were advised of the procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent. Two to 4 mL of aspirate was drawn into heparinized tubes, diluted in phosphate-buffered saline (PBS), layered over Ficoll-Hypaque gradients (density, 1.077 g/mL), and centrifuged at 400 g for 30 minutes, and the interface buoyant mononuclear cells (MNC) were collected and washed three times in Iscove's modified Dulbecco's medium (IMDM). These cells are subsequently referred to as MNC. M ϕ were removed by two consecutive (45 minutes each) adherence procedures at 37 °C in polystyrene tissue culture dishes using IMDM with 7.5% fetal calf serum (FCS) at cell concentrations of 10⁷ cells/mL.^{11,12} Nonadherent buoyant cells (NAB) obtained in this manner contained <2% M ϕ by esterase stain and immunofluorescent labeling with LeuM3 monoclonal antibody (MCAB). Adherent MNC (>95% M ϕ by esterase staining and LeuM3 MCAB immunofluorescence), were isolated by gentle scraping with a rubber policeman after vigorous washing to remove the loosely bound cells.

T cells were removed from the marrow by solid-phase immunoabsorption "panning."¹¹ In this method, polystyrene Petri dishes were coated with 100 μ g of affinity-purified goat antimouse IgG (Tago, Burlingame, CA) diluted in 10 mL of 0.05 mol/L TRIS buffer. After a 40-minute incubation at 23 °C, the buffer was decanted and the dishes gently washed five times with PBS. Cell suspensions (20 $\times$ 10⁶ cells in 75 μ L IMDM) were coated with 50 to 100 μ g of Leu1 pan-T MCAB, incubated at 4 °C for 20 minutes, centrifuged, and then added to the antibody-coated dish (indirect panning) and incubated for 70 minutes at 4 °C. Cells bound to the coated dish and nonbound cells were separately recovered by differential elution with cold PBS-1% FCS. In almost all cases, >95% of the bound cells and <2% of the nonbound cells stained positively by cytofluorographic analysis with a combination of two or three separate (Leu1, Leu4, and Leu5) pan-T MCABs. In control experiments, cultures of M ϕ or T cells alone never yielded >2% BFU-E per 10⁵ cells plated.¹²

Assay systems for hematopoietic progenitor cells. Marrow MNCs (0.5 to 1.5 $\times$ 10⁵/mL) were cultured with 0.5 U/mL of human urinary Ep (1,140 National Institutes of Health [NIH] 1,140 U/mg protein) in triplicate 0.25-mL aliquots containing 1.1% methylcellulose, 50 μ mol/L 2-mercaptoethanol, and 15% to 30% FCS with or without 1% bovine serum albumin (BSA) and MoCM-conditioned medium (MoCM, a medium conditioned by a T lymphoblast cell line containing both colony-stimulating activity and erythroid burst-promoting activity [BPA]¹³). Cells were incubated at 37 °C and 5% CO₂ in either 21% O₂ (room air) or 5% O₂ in modular chambers (Billips & Rothenberg, Del Mar, CA). Colonies were scored on days 12 to 14 to assess committed erythroid (BFU-E) progenitor cells as previously described.¹²

The percent augmentation of BFU-E growth was calculated based upon the following formula:

$$\text{Percent augmentation} = \frac{(\text{no. colonies at 5\% O}_2) - (\text{no. colonies at 21\% O}_2)}{(\text{no. colonies at 21\% O}_2)}$$

In some experiments, enriched populations (1 to 10 $\times$ 10⁴ cells/mL) of either marrow M ϕ or marrow T cells were added back to cultures containing 1 to 2 $\times$ 10⁵ autologous marrow MNCs previ-

ously depleted of monocytes and/or T lymphocytes. Control groups, concurrently assessed for progenitor cell growth, included enriched marrow T cells or M ϕ alone and unfractionated marrow MNCs; the cultures were grown separately but simultaneously and assayed in the presence and absence of Ep $\pm$ MoCM for the growth of marrow BFU-E.

Establishment and monitoring of oxygen tensions in cultures. Low (5%) O₂ cultures were set up using gas-tight modular chambers that can be flushed with any desired gas mixture. Multiwell tissue culture plates were placed in modular chambers and flushed with 5% O₂, 5% CO₂, and 90% N₂ for five minutes. Plates filled with sterile water were placed in the modular chambers to provide humidity. The residual O₂ in multiwell plates was allowed to equilibrate with the gas phase in the chambers for one to two hours, after which the modular chambers were flushed once again with the 5% O₂ gas mixture. The pO₂ and pCO₂ in the chambers were monitored daily and maintained at 5% $\pm$ 0.5% for 14 days of culture. Control cultures were maintained at 21% O₂ in modular chambers by flushing with a gas mixture of room air with 5% CO₂.

Preparation of CM. Subpopulations of purified bone marrow T lymphocytes or M ϕ were incubated in IMDM with 50 μ mol/L 2-mercaptoethanol at either 21% O₂ or 5% O₂ for two days at a concentration of 2 $\times$ 10⁵ cells/mL. CM were also prepared from a mixture of M ϕ and T cells at M ϕ :T ratios of 1:6 (total cells, 2 $\times$ 10⁵). CM supernatants were harvested, spun at 1,800 g for ten minutes, and either used on the day of harvest or stored at 4 °C until testing. CM were assessed at 12% vol/vol against marrow MNCs depleted of both M ϕ and T cells (NAB-T).

Assessment of CM for BPA. To improve the detection of BPA in these CM, we plated NAB-T cells in 30% FCS and 1% BSA in the presence of CM and delayed the addition of Ep to cultures until day 3.¹⁴ Negative controls consisted of wells plated in the presence of IMDM but in the absence of any added CM, and the addition of 1% MoCM was used as a positive control. In control experiments, 10 $\pm$ 3 BFU-E and 2 $\pm$ 2 CFU-GM were formed per 10⁵ cells in the absence of MoCM, whereas 118 $\pm$ 9 BFU-E and 142 $\pm$ 7 CFU-GM were formed in the presence of 1% MoCM.

Effects of antioxidants on BFU-E at 21% and 5% O₂. The effects of antioxidants on BFU-E were assessed by culturing bone marrow MNCs in the presence and absence of 0 to 10 mmol/L reduced glutathione (Sigma Chemical Company, St Louis), 0.1 to 1,000 μ g/mL of superoxide dismutase (Biotics Research Corp, Houston), or 0 to 100,000 U/mL of catalase (Sigma) at both 21% and 5% O₂. Control experiments using superoxide dismutase in a system containing acetaldehyde-xanthine oxidase plus reduced cytochrome c indicated that the superoxide dismutase used in our culture system was an efficient superoxide scavenger. Similar concentrations of antioxidants have been used to successfully inhibit free radical-induced damage to red cell membrane and to inhibit up to 90% of hydroxyl radical generation by human phagocytes.¹⁵ Glutathione was always freshly prepared for each assay.

Assessment of erythroid progenitor sensitivity to BPA under varying culture oxygen tensions. We assessed the differential sensitivity of marrow BFU-E to exogenous BPA (MoCM) at 21% and 5% O₂. Cultures (n = 4) were established with marrow target cells depleted of both M ϕ and T cells, in 15% FCS in the absence of BSA, and in the presence of 0% to 10% MoCM. In control experiments performed at both 21% and 5% O₂, the addition of 0.2% to 1.0% MoCM produced a three to fivefold increment in marrow BFU-E. The percent change in BFU-E under 5% O₂ relative to 21% O₂ was calculated at each concentration of MoCM. Both the absolute number of BFU-E and the percent change observed at 5% O₂ relative to 21% O₂ were compared at MoCM concentrations of 0% to 10%.

Statistics. The two-sample unpaired Student's *t* test was used in statistical analysis of the data.¹⁶

Table 1. Augmentation in Human Marrow BFU-E at 5% O₂

Ep (U/mL)	No. BFU-E per 100,000 Cells*										Percent Change †
	21% O ₂	5% O ₂	21% O ₂	5% O ₂	21% O ₂	5% O ₂	21% O ₂	5% O ₂	21% O ₂	5% O ₂	
0.1	84	122	163	215	47	67	71	111	32	67	44.0 ± 4.5
0.2	138	226	187	267	32	64	97	147	72	115	62.0 ± 12.3
0.5	219	326	175	336	72	123	125	138	112	163	54.8 ± 7.9
1.0	182	250	232	347	96	138	103	120	170	199	33.0 ± 6.1
1.5	163	248	—	—	134	210	91	128	136	195	39.8 ± 8.0

Each value represents the mean ± SEM of results from five separate experiments, each involving a different normal marrow donor. Colony numbers are indicated for only three representative experiments to simplify the table.

*Mean value for BFU-E per 10⁵ marrow mononuclear cells; each experiment is plated in triplicate.

†Percent change = (no. BFU-E at 5% O₂ - no. BFU-E at 21% O₂) × 100/no. BFU-E at 21% O₂.

RESULTS

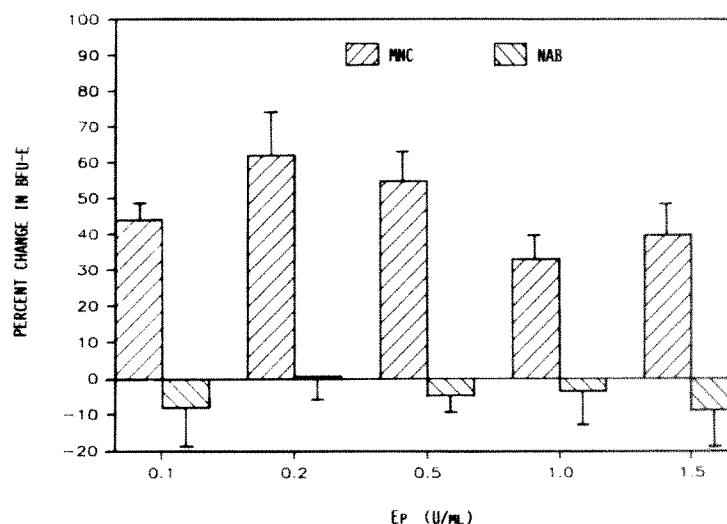
Augmentation of marrow erythropoiesis at 5% O₂. Physiological oxygen tensions (5% O₂) augmented in vitro bone marrow erythropoiesis relative to marrow cultures grown at 21% O₂. The increase in erythropoiesis, as detected by an augmentation in the number of human burst-forming units (BFU-E) grown from marrow MNC, was observed over a wide range of Ep concentrations (0.1 to 1.5 U/mL) (Table 1 and Fig 1). Table 1 lists five experiments in which BFU-E augmentation is demonstrable at each Ep concentration used along with the mean BFU-E augmentation. There was a 33% to 62% (n = 5) increase in BFU-E when marrow MNC were cultured at 5% O₂ compared with cultures established at room air (21% O₂) ($P < .001$). This increase appeared to be independent of Ep. No erythroid colonies were detected under 5% O₂ in the absence of added Ep (data not shown). The increase in BFU-E at 5% O₂ was not significantly ($P > .05$) diminished by the addition of MoCM, a potent source of exogenous BPA; in nine experiments, a 39% ± 10% increase in BFU-E was observed at 5% O₂ (over 21% O₂) in the presence of 0.5 U/mL of Ep plus 1% MoCM compared with a 55% ± 8% increase in BFU-E when paired, autologous marrow cells were concomitantly grown in the presence of 0.5 U/mL of Ep alone. The increase in BFU-E was maintained even in the presence of 2% to 10% MoCM (data not shown). In control experiments, the maximal BFU-E expression was observed between 0.5% and 2.0% MoCM (data not shown).

Effect of Mφ and/or T cell depletion on augmentation of marrow erythropoiesis under 5% O₂. Depletion of Mφ from marrow MNC (NAB) abolished the augmentation in BFU-E growth under 5% O₂ over the entire range of Ep concentrations studied (Fig 1) ($P < .001$). Similarly, no augmentation was observed under 5% O₂ when nonadherent marrow cells were cultured in the presence of MoCM plus Ep (Fig 2). Augmentation of erythropoiesis was also abolished when marrow MNC were depleted of T lymphocytes. Marrow T cell depletion (MNC-T) abrogated erythropoietic stimulation whether cultures were established with Ep alone or with MoCM plus Ep (Fig 2). No augmentation in erythropoiesis was noted at 5% O₂ relative to 21% O₂ when marrow MNC were depleted of both monocytes and T cells (NAB-T) and cultured in the presence of Ep alone or MoCM plus Ep (Fig 2).

Augmentation of CFU-GM at 5% O₂. Physiological (5%) O₂ tensions also augmented CFU-GM growth (186% ± 64% increase over control) from marrow MNC relative to marrow MNC grown under 21% O₂ (Table 2). However, the removal of T cells (110% ± 17% increase over control), Mφ (133% to 54% increase over control), or both T cells and Mφ (106% ± 26% increase over control) did not abolish the granulopoietic augmentation under 5% O₂ (Table 2).

Lack of BFU-E augmentation at 5% O₂ following the addition of Mφ or T cells to autologous, nonadherent T cell-depleted marrow MNC. We assessed BFU-E growth at 5% and 21% O₂ following the addition of either 0% to 20%

Fig 1. Augmentation in erythroid progenitors (BFU-E) cultured from human bone marrow MNC at various Ep concentrations and the effect of marrow MNC depletion on erythropoietic augmentation at 5% O₂. Results are expressed as means ± SEM of the percent change in BFU-E observed at 5% O₂ compared with 21% O₂ (see Materials and Methods) and are derived from five to nine separate experiments. At 0.5 U/mL Ep in a representative experiment, 175 and 336 BFU-E/10⁵ are obtained at 21% O₂ and 5% O₂, respectively, with marrow MNC, whereas 190 and 182 BFU-E/10⁵ are obtained at 21% O₂ and 5% O₂, respectively, with marrow NAB.



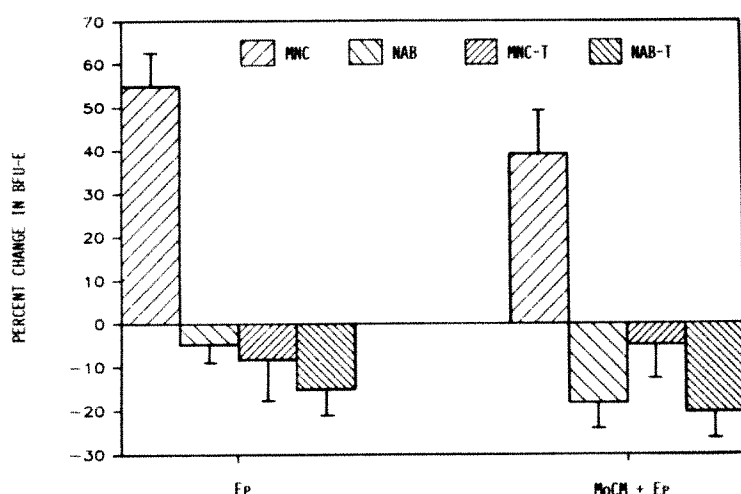


Fig 2. Effect of M ϕ , T cells, and exogenous BPA (MoCM) on erythropoietic augmentation at 5% O₂. Human bone marrow MNC were plated before and after depletion of monocytes (NAB) by two consecutive adherence separation procedures, after depletion of T cells with immunoabsorbant panning with Leu1 pan-T MCAb (MNC-T), or after depletion of both Mo and T cells (NAB-T) by a double-adherence procedure followed by panning. Results are expressed as means $\pm$ SEM of the percent change in BFU-E observed at 5% O₂ compared with those at 21% O₂ (see Materials and Methods) and are derived from seven to ten separate experiments.

T cells or 0% to 20% M ϕ to autologous marrow MNC depleted of both Mo and T cells. As demonstrated in Table 3, the addition of 0% to 20% T cells to nonadherent, T cell-depleted marrow MNC failed to restore erythropoietic augmentation at 5% O₂ ($P > .5$); no BFU-E augmentation was achieved even after the addition of 40% T cells (data not shown). Similarly, the addition of 10% to 20% autologous M ϕ to M ϕ -free, T-depleted marrow MNC also failed to restore augmentation of erythropoiesis under 5% O₂ ($P > .5$) (Table 3).

Augmentation of BFU-E under 5% O₂ following the addition of M ϕ to nonadherent marrow MNC or the addition of T cells to T cell-depleted marrow MNC. Augmentation of marrow erythropoiesis under 5% O₂ was restored by reconstituting nonadherent, T cell-depleted marrow MNC with both autologous M ϕ and T cells. Thus, the addition of 5% to 50% autologous M ϕ to M ϕ -free marrow MNC resulted in a 26.2% to 43.0% ($P < .01$) increase in BFU-E under 5% O₂ compared with control M ϕ -repleted cultures established from the same donor but grown under 21% O₂ (Table 4). Similarly, the addition of 10% T cells to autologous, T cell-depleted marrow MNC augmented erythropoiesis under 5% O₂ by 56.2% $\pm$ 14.1% ($P < .01$). The addition of 10% M ϕ plus 10% T cells to autologous, nonadherent T cell-depleted marrow target cells also restored BFU-E augmentation under 5% O₂ (-4.3% $\pm$ 10.6% to 76.2% $\pm$ 10.5%) ($P < .001$). These results, in toto, indicate that both M ϕ and T cells are required for erythropoietic augmentation at 5% O₂.

Effects of CM prepared from purified T cells and M ϕ at 5% or 21% O₂ on BFU-E growth from NAB-T. To confirm the role of T cells and monocytes in mediating BFU-E augmentation under 5% O₂, we prepared two-day CM from marrow M ϕ and/or T cells at 5% O₂ and 21% O₂ and assessed these CM for BPA against nonadherent, T cell-depleted marrow MNC. We delayed the addition of Ep to cultures until day 3 to increase the sensitivity of the BPA assay by eliminating Ep-sensitive BFU-E. Under these conditions, only minimal numbers ($10 \pm 3/10^5$ NAB-T) of BFU-E were obtained in the absence of MoCM; a substantial increase ($118 \pm 9/10^5$ cells plated) was observed upon the addition of exogenous BPA (1% MoCM). CM prepared from either T cells or M ϕ alone did not provide erythroid potentiating activity at either 5% or 21% O₂ (Fig 3). However, CM prepared under 5% O₂ from T cell-M ϕ mixtures provided potent ($P < .001$) BPA activity compared with identical M ϕ -T cell mixtures prepared under 21% O₂ (Fig 3) ($n = 4$). CM prepared for 24 hours from mixtures of T cells and M ϕ under 5% and 21% O₂ and assessed against autologous marrow NAB-T target cells gave similar results in which only CM prepared with T cell-M ϕ mixtures under 5% O₂ provided potent BPA activity. The low erythroid potentiation obtained from T cell-M ϕ CM established under 21% O₂ most likely reflects the short preparation time of the CM (48 hours) as well as the relatively low number (2×10^5 cells/mL) of cells used in CM preparation.

Assessment of these CM for CFU-GM showed low CSF-GM activity. In the absence of exogenous CSF, only 2 ± 1

Table 2. Augmentation of Human Marrow CFU-GM at 5% O₂

	No. CFU-GM/100,000 Cells*						Percent Change
	21% O ₂	5% O ₂	21% O ₂	5% O ₂	21% O ₂	5% O ₂	
MNC	56	108	51	135	27	83	186 $\pm$ 64†
NAB	73	113	76	115	40	117	133 $\pm$ 54
MNC-T	65	180	—	—	28	65	110 $\pm$ 17
NAB-T	51	140	68	131	—	—	106 $\pm$ 26

Each value represents the mean $\pm$ SEM of results from seven to nine separate experiments, each involving a different normal donor. Paired autologous marrow samples were cultured with Ep + MoCM. Colony numbers are indicated for only three representative experiments to simplify the table.

*Mean value for CFU-GM per 10^5 marrow MNC; each experiment is plated in triplicate.

†Percent change = (no. CFU-GM at 5% O₂ - no. CFU-GM at 21% O₂) $\times$ 100/no. CFU-GM at 21% O₂.

Table 3. Effect of the Addition of Autologous M ϕ or T Lymphocytes (T) on BFU-E Grown Under Either 21% O₂ or 5% O₂ From Nonadherent, T Cell-Depleted Marrow Cells

T (%)	Mo (%)	Exp 1		Exp 2		Exp 3		Percent Change BFU-E*
		21% O ₂	5% O ₂	21% O ₂	5% O ₂	21% O ₂	5% O ₂	
0	0	340	304†	83	67	107	121	-8.9 ± 0.1‡
5	0	315	278			124	147	-10.3 ± 10.3
10	0	235	258			116	118	-10.3 ± 13.3
20	0	256	287	102	112			3.0 ± 1.0
0	10			138	135	96	91	1.1 ± 2.2
0	20			134	130	118	118	-3.5 ± 1.0

BFU-E were cultured in the presence of 0.5 U/mL of Ep.

*Percent change = (no. BFU-E at 5% O₂ - no. BFU-E at 21% O₂) × 100/no. BFU-E at 21% O₂.

†Mean erythroid progenitor (BFU-E) number per 10⁵ nonadherent, T cell-depleted marrow MNC; each experiment was plated in triplicate and involved a separate normal donor.

‡Each value represents the mean ± SEM of results from four separate experiments involving a different normal donor. Only three representative experiments are indicated in the Table.

Table 4. Effect of the Addition of Autologous T Cells and/or M ϕ on BFU-E Grown From NAB, MNC-T, and NAB-T Marrow Target Cells

BM Cells	Mo (%)	T (%)	Exp 1		Exp 2		Exp 3		Percent Change in BFU-E*	P Value†
			21%	5%	21%	5%	21%	5%		
NAB	0	—	98	104‡	130	126	91	99	2.9 ± 5.5	>.2
NAB	5	—	102	119	128	171			26.2 ± 5.0	<.001
NAB	10	—	110	131	112	175	160	211	36.7 ± 13.6	<.001
NAB	20	—	88	112			183	242	43.5 ± 19.0	<.001
NAB	50	—			150	212			43.0 ± 0.7	<.025
MNC-T	—	0	87	103	163	150	146	163	-5.1 ± 7.6	>.1
MNC-T	—	10	116	151	116	155	94	187	56.0 ± 14.1	<.001
NAB-T	0	0	88	69	69	84	45	40	-4.3 ± 10.6	>.5
NAB-T	10	10	83	126	73	132	56	111	76.2 ± 10.5	<.001

Percent change = (no. BFU-E at 5% O₂ - no. BFU-E at 21% O₂) × 100/no. BFU-E at 21% O₂.

*Each value represents the mean ± SEM of results from three to five separate experiments, each involving a different normal donor.

†P values were determined with the paired Student's *t* test from the triplicate BFU-E values of three to five experiments.

‡Mean value for BFU-E per 10⁵ marrow cells; each experiment was plated in triplicate.

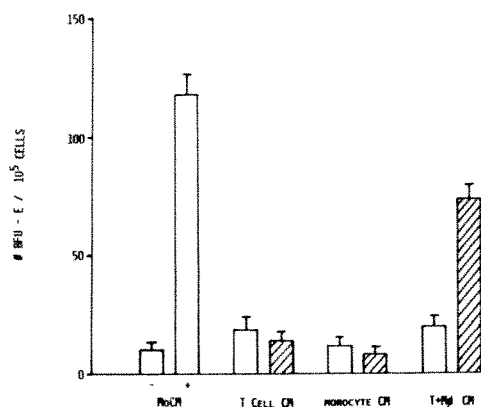


Fig 3. Effect of CM prepared at 5% or 21% O₂ on erythroid progenitors (BFU-E) cultured from nonadherent, T cell-depleted target cells. CM were prepared for one to two days under either 5% or 21% O₂ using either marrow M ϕ (2×10^5 cells/mL) or T cells (2×10^5 cells/mL) alone or mixtures of both M ϕ and T cells (total, 2×10^5 cells/mL). CM (12%, vol/vol) were assessed against NAB-T target cells. Results are expressed as means ± SEM from four experiments. Ep, 5 U/mL added D3, no MoCM except for panel 2. □, 21% O₂; ▨, 5% O₂.

CFU-GM was observed, which increased to 142 ± 7 CFU-GM upon the addition of 1% MoCM. CM from T cell-M ϕ mixtures under 21% O₂ gave rise to 6 ± 1 CFU-GM, whereas CM from a T cell/M ϕ mixture under 5% O₂ yielded 21 ± 3 CFU-GM.

Effects of antioxidants on BFU-E growth at 21% and 5% O₂. To determine whether the increased colony-forming ability of bone marrow MNC at 5% O₂ reflects the protection of erythroid progenitors from accessory cell-derived oxidant injury at 21% O₂, we cultured marrow target cells containing T cells and M ϕ in the presence of three separate antioxidants: reduced glutathione, an intracellular antioxidant, at a concentration range of 0.1 μ mol/L to 10 mmol/L; superoxide dismutase, a scavenger for superoxide radicals, at a concentration range of 0.1 to 1,000 μ g/mL; and catalase, which reacts with H₂O₂, at a concentration range of 1 to 100,000 U/mL. Cultures were established at both 21% and 5% O₂. As shown in Figs 4A, B, and C, marrow BFU-E grown in the presence of varying concentrations of the three antioxidants failed to demonstrate augmented growth at 21% O₂, which suggests that the observed augmentation in BFU-E grown from marrow MNC at 5% O₂ in the absence of antioxidants did not arise as a consequence of reduced O₂ toxicity. Moreover, augmentation in BFU-E was maintained at 5% O₂ relative to 21% O₂ in the presence of each of these antioxidants.

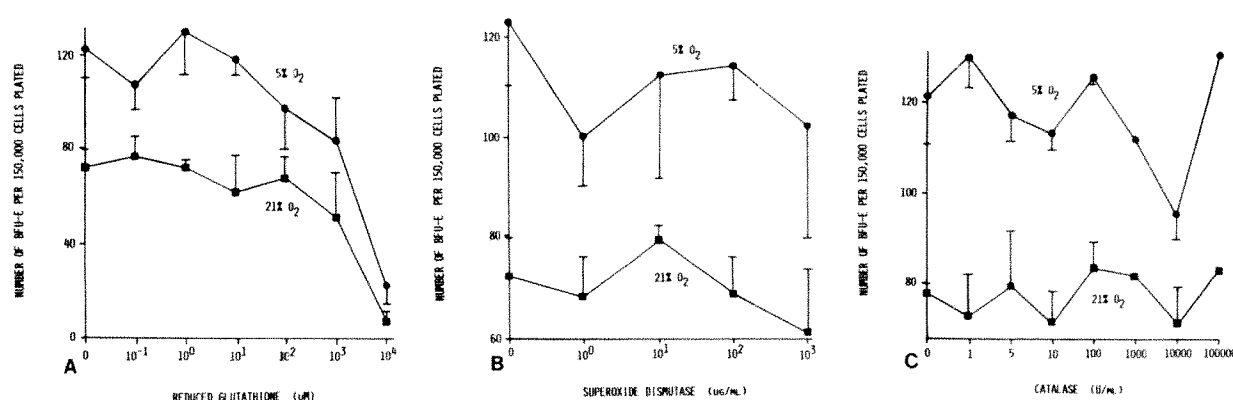


Fig 4. Effect of antioxidants on erythroid progenitors (BFU-E) grown from unseparated bone marrow MNC at 5% and 21% O₂. Results are expressed as means $\pm$ SEM of the number of BFU-E per 1.5×10^5 cells plated from three separate experiments. (A) Reduced glutathione. (B) Superoxide dismutase. (C) Catalase.

Sensitivity of BFU-E to BPA under 5% O₂. Although the aforementioned results indicate that M ϕ and T cells together mediate augmentation of BFU-E under 5% O₂, we next assessed whether low oxygen tensions may additionally alter the sensitivity of early erythroid marrow progenitors to BPA. Using nonadherent, T cell-depleted marrow target cells, we assessed the differential sensitivity of BFU-E cultured at 5% O₂ or 21% O₂ to varying concentrations of exogenous BPA (MoCM). Figure 5A shows the results of a representative experiment of dose-response curves measured at 5% and 21% O₂ as a function of MoCM concentration. The dose-response curves at the two O₂ concentrations were virtually identical. The results of three additional experiments confirmed this observation (data not shown). Any change in the sensitivity of BFU-E under 5% O₂ to exogenous BPA would be reflected in augmented colony growth at low MoCM concentrations, especially since similar number of BFU-E were obtained at 5% and 21% O₂ in the absence of MoCM. However, our results indicate that the percent change in BFU-E growth for 1% to 10% MoCM fluctuates around zero ($n = 4$) (Fig 5B). These results suggest that

marrow BFU-E cultured in the presence of either 5% O₂ or 21% O₂ demonstrate similar sensitivity to exogenous BPA.

DISCUSSION

We have investigated the effects of physiological O₂ tensions (5% O₂) on human marrow BFU-E growth. Our results demonstrate augmentation in BFU-E when marrow MNC are grown under 5% O₂. BFU-E enhancement appears to be mediated by M ϕ and T cells, since the removal of either M ϕ , T cells, or both abolished BFU-E augmentation at 5% O₂. The addition of 10% to 20% M ϕ or 5% to 40% T cells to autologous nonadherent T cell-depleted marrow target cells failed to restore BFU-E augmentation at 5% O₂. However, the readdition of M ϕ to nonadherent MNC, T cells to T cell-depleted MNC, or both T cells and M ϕ to nonadherent T cell-depleted target cells restored BFU-E augmentation under 5% O₂. CM prepared at 5% O₂ from a mixture of M ϕ and T cells demonstrated potent BPA activity, whereas little or no BPA activity was detected with CM from a mixture of T cells and M ϕ at 21% O₂ or from T cells or M ϕ alone under either 5% or 21% O₂. No BFU-E augmentation was observed

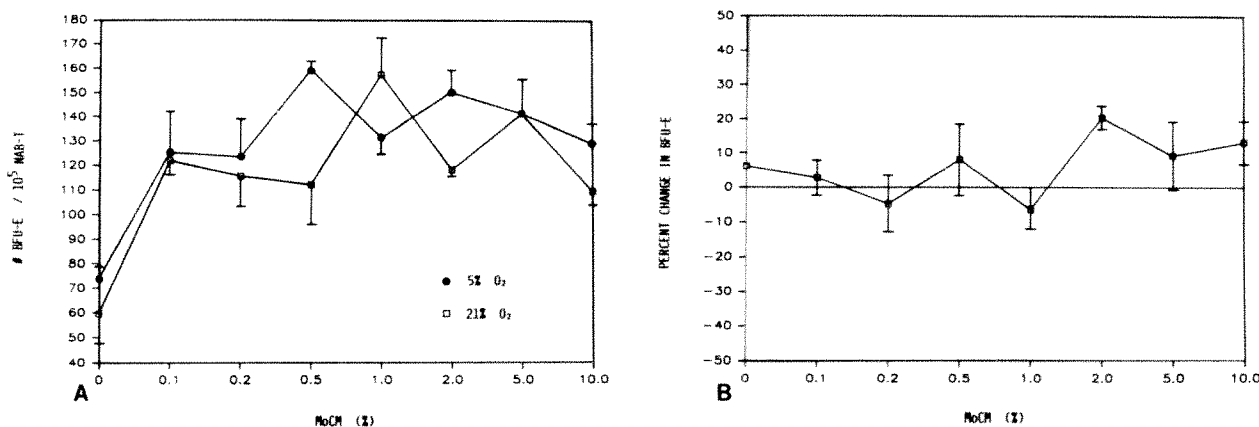


Fig 5. Sensitivity of erythroid progenitor cells (BFU-E) grown under either 5% O₂ or 21% O₂ to exogenous BPA derived from MoCM. (A) BPA dose-response curves under 5% and 21% O₂. BPA dose-response curves were determined using nonadherent T cell-depleted (NAB-T) marrow target cells and 0% to 10% MoCM as a source of exogenous BPA. Bone marrow cells were grown under either 21% O₂ or 5% O₂ and were obtained from the same donor. Results are expressed as means $\pm$ SEM from triplicate cultures. (B) Percent augmentation under 5% O₂ as a function of BPA concentration ($n = 4$). Note that the percent change fluctuates around 0.

under 21% O₂ when BFU-E were cultured in the presence of a variety of antioxidants. The sensitivity of BFU-E grown from nonadherent T cell-depleted marrow target cells to exogenous BPA was virtually identical at 5% O₂ and 21% O₂. These results suggest that T cells and M ϕ collaborate to produce BPA and augment human marrow erythropoiesis under 5% O₂.

Although CFU-GM growth was augmented at 5% O₂ relative to 21% O₂, it was not mediated by T cells and M ϕ , since the removal of either T cells or M ϕ did not abolish the CFU-GM augmentation. In addition, CM prepared from T cell/M ϕ mixtures at 5% O₂ demonstrated little CSF activity. Enhancement in colony formation by T cells and M ϕ under 5% relative to 21% O₂ is therefore specific for BFU-E.

A reduction in oxygen toxicity under physiological O₂ tensions may contribute to augmented BFU-E growth at 5% O₂. Rich and Kubanek have observed augmented growth of murine CFU-E and BFU-E under reduced O₂ tensions and have demonstrated that part of this increase in erythropoiesis can be explained by lowered oxygen toxicity under 5% O₂.⁷ Direct oxygen toxicity affecting erythroid progenitor cells with a consequent impairment of BFU-E expression at 21% O₂ seems unlikely from our experimental results, since BFU-E growth from marrow target cells depleted of both M ϕ and T cells was not augmented under 5% O₂. Impairment of T cell-M ϕ collaboration as a consequence of oxygen toxicity at ambient (21% O₂) concentrations could explain the observed BFU-E augmentation under 5% O₂. If this were the case, we would most likely have observed erythropoietic augmentation with marrow MNC containing M ϕ and T cells in the presence of antioxidants. No such stimulation was observed when BFU-E were cultured in the presence of either reduced glutathione, superoxide dismutase, or catalase, which suggests that reduced O₂ toxicity at lower O₂ tensions does not account for the BFU-E augmentation in the absence of antioxidants at 5% O₂. Moreover, BFU-E augmentation at 5% O₂ relative to 21% O₂ is maintained in the presence of each of the assessed antioxidants, which suggests that the observed augmentation is independent of O₂ toxicity. Finally, Ascensao¹⁷ has shown that oxidant injury to human erythroid progenitor cells can be demonstrated only in the presence of low serum concentrations. In addition, Shulman et al¹⁸ demonstrated oxygen toxicity to human erythroid progenitor cells only in the presence of a superoxide-generating system like xanthine-xanthine oxidase. Since we routinely used 50 μ mol/L 2-mercaptoethanol and 30% FCS in our culture system, the role of O₂ toxicity in mediating erythropoietic augmentation under 5% O₂ may well not be significant.

To our knowledge, this is the first demonstration that the augmented BFU-E growth under low O₂ tensions is mediated directly or via additional accessory cells by both M ϕ and T cells. Several reports in the literature suggest that either T cells, M ϕ , or both are required for BFU-E and CFU-GM proliferation *in vitro* at ambient O₂ tensions.^{12,19-29} However, the uniqueness of our results is that T cells and M ϕ can mediate augmented erythropoiesis by releasing burst-enhancing factors in response to variations in O₂ tensions.

This is an important concept since there is a gradation in O₂ tensions in the circulation, capillaries, in various tissues, and in bone marrow medullary cavities.¹⁻³ Increased erythropoietin production or release is considered the sole mechanism mediating erythropoietic response to low O₂; our results suggest that immunoregulatory mononuclear cells can also sense variations in O₂ tensions by releasing hematopoietic growth-promoting activities. Whether M ϕ in response to variations in O₂ tensions release a factor similar to that described by Bagby et al²⁷ that stimulates BPA production by T cells or whether T cells in response to variations in O₂ tensions stimulate M ϕ either directly or through a soluble mediator²⁰ remains to be elucidated.

There are precedents in the literature for low O₂ tensions affecting specific cellular metabolic activities and the ability of MNC to secrete or respond to purified growth factors. O₂ has been demonstrated to alter activities of several enzymes that require molecular O₂ for activity (eg, cytochrome oxidase and ATPase). Simon et al and Cummisley et al have demonstrated in murine epithelial cells and macrophages that enzyme activities in the glycolytic pathway (eg, pyruvate kinase, phosphofructokinase, and hexokinase) are elevated and those of oxidative phosphorylation (eg, cytochrome oxidase) are depressed under 2% O₂.^{30,31} Knighton et al have recently demonstrated that when cultured under low pO₂ macrophages secrete an active angiogenesis factor that aids wound healing.³² The factor secretion ceased when cells were returned to ambient O₂ tensions; thus in this example, MNC appear to secrete a growth factor in response to low O₂ tensions. Smith et al (personal communication) have demonstrated that proliferative response to the growth hormone IgF1 by IgF1 receptor-positive T leukemia cells is achieved only under 5% O₂. In this example, low O₂ tensions enable the T cells to respond to IgF1.

Monocytes have been reported to release hematopoietic growth factors under reduced O₂ tensions in murine and human systems. Rich et al have demonstrated that murine monocytes, when cultured in hydrophobic Teflon foils, can be induced to release Ep, BPA, and CSF-GM at 21% O₂ and that the release of Ep can be augmented at 5% O₂.^{33,34} It is unlikely that the erythroid augmentation observed at 5% O₂ in our experiments is mediated by Ep, since no BFU-E were formed at 5% O₂ in the absence of Ep and marrow BFU-E augmentation is maintained even at high concentrations of Ep (Fig 2). Broxmeyer⁸ has also observed, in a preliminary report, an increased release of CSF-GM from peripheral blood M ϕ under low O₂ tensions, which suggests a role for accessory cells in mediating granulopoietic augmentation under 5% O₂.

Although M ϕ and T cells augment BFU-E at 5% O₂ by collaborating to produce BPA-like activity, the addition of excess exogenous BPA (MoCM, 1% to 10%) did not abrogate this augmentation. The failure of MoCM to abrogate erythroid augmentation under 5% O₂ suggests a possible heterogeneity in either the progenitor target cell population and/or in the erythroid potentiating responses of T cells and M ϕ . Alternatively, progenitor cells may well be capable of responding to greater amounts of erythroid potentiating

activity than that provided by MoCM. MoCM is prepared from an HTLV-containing leukemic T cell line and undoubtedly contains a mixture of lymphokines in addition to BPA, some of which may suppress erythropoiesis.³⁵ Third, T cells and M ϕ may collaborate at 5% O₂ tensions to release potentiators of erythropoiesis distinct from BPA. Prostaglandin E, which is released by M ϕ and stimulates BFU-E in the presence of T cells, is a possible mediator of augmented BFU-E at 5% O₂.³⁶⁻³⁸ However, in two separate experiments, we have observed that the BFU-E augmentation observed under 5% O₂ is maintained in the presence of up to 10 μ mol/L indomethacin, a potent inhibitor of prostaglandin E release from M ϕ .³⁸ Interleukin 2 (IL2) is another possible T cell-derived lymphokine capable of erythropoietic augmentation under ambient O₂ tensions,³⁹ and it may have a role in mediating the BFU-E response to physiological (5%) O₂ tensions. However, although IL2 can stimulate BFU-E proliferation in the presence of BPA and accessory cells, the role of IL2 in BFU-E proliferation in the absence of accessory cells and BPA is not yet established. Finally, it is possible that a suppressor lymphokine for erythropoiesis is released via a collaborative interaction between T cells and M ϕ at 21% O₂ but not at 5% O₂. We rule out this possibility based on the following observations: (1) The number of BFU-E with CM derived from T cell-M ϕ mixtures at 21% O₂ was not lower than in the presence of CM derived from either T cells or M ϕ alone. (2) The addition of T cell/M ϕ CM (at 21% O₂) to cultures containing MoCM did not reduce BFU-E growth (data not shown), and (3) CM from T cell/M ϕ

mixtures contained little interferon activity (1 to 2 U/mL as measured by radioimmunoassay), either at 5% or 21% O₂.

Broxmeyer has reported increased human marrow CFU-GM growth under 5% O₂ when compared with simultaneous cultures established under 21% O₂.⁸ This augmentation was accompanied by an increased HLA-DR expression on CFU-GM progenitor cells and by an increased number of S-phase progenitor cells. Although any direct effect of altered O₂ tensions on the erythroid progenitor cell independent of T cells and Mo appears unlikely from our data, it is possible that the BPA-like activity released by T cell-M ϕ collaboration under 5% O₂ may induce increased HLA-DR expression on erythroid progenitor cells and may increase the percentage of erythroid progenitor cells in S-phase.

Our data suggest that T cells and M ϕ can respond to variations in O₂ tensions by collaborating, either directly or indirectly, to produce BPA-like activity with a consequent augmentation in erythropoiesis. Although it has been well documented that increased erythropoietin is produced in response to in vivo hypoxic stress,⁴ we speculate that augmentation in the production or release of BPA-like activity from M ϕ and T cells could represent an additional mechanism mediating the response of erythropoiesis to alterations in tissue and marrow oxygen tensions. Preliminary experiments in our laboratory⁴⁰ indicate that the lack of appropriate compensatory polycythemia in some patients with chronic disease and hypoxemia may be, in part, a consequence of impaired progenitor augmentation under reduced oxygen tensions.

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T Cell Differentiation Stages Identified by Molecular and Immunologic Analysis of the T Cell Receptor Complex in Childhood Lymphoblastic Leukemia

By Joseph Mirro, Jr, Geoffrey Kitchingman, Frederick G. Behm, Sharon B. Murphy, and Rakesh M. Goorha

T cell differentiation was investigated by determining the relationship of T cell receptor (Ti) gene rearrangement and transcription to the expression of surface and cytoplasmic T3 antigen using blast cells from five children with acute lymphoblastic leukemia of thymic origin. Patterns of monoclonal antibody (MoAb) reactivity indicated that these cases were representative of the three recognized stages (I, II, III) of human thymocyte development. The T3 antigen, which becomes linked to the Ti to form a functional T cell receptor complex on mature thymocytes, was expressed on the cell surface in two cases (stage III). However, in the remaining three cases that were surface T3 negative (stages I and II), large amounts of T3 were identified in the cytoplasm by immunoperoxidase staining and flow cytometry. Leukemic blasts from all five patients showed rear-

ranged genes encoding the β -chain portion of the Ti heterodimer. RNA transcripts of Ti β -chain genes were also evident in lymphoblasts from all five cases, but transcripts coding for the α -chain portion of Ti were found only in cases that expressed T3 on the cell surface. Thus the absence of surface T3 (and presumably Ti) coincides with the absence of Ti α -chain RNA, suggesting that transcription of α -chain genes is a critical regulatory event in the surface expression of the Ti-T3 complex. Leukemic T cells that rearrange and express Ti β -chain genes but lack Ti α -chain messenger RNA (mRNA) may represent a stage of differentiation analogous to pre-B cells, where heavy-chain immunoglobulin (Ig) genes are rearranged and expressed but light-chain Ig genes are not expressed.

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THREE STAGES of T cell ontogeny have been delineated using monoclonal antibodies (MoAbs) to detect the expression of the cell surface antigens T11, T6, T4, T8, and T3. Stage I is characterized by the phenotype T11⁺, T6⁻, T4⁻, T8⁻, T3⁻; stage II by T11⁺, T6⁺, T4⁺, T8⁺, T3⁻; and stage III by T11⁺, T6⁻, T4⁺, T8⁺, T3⁺.¹ A 90-kDa disulfide-linked heterodimer, composed of α and β subunits and termed the T cell antigen receptor Ti, also appears on the surface of stage III T lymphocytes and is responsible for T cell antigen specificity.²⁻⁵ The ability of the Ti molecule to recognize many diverse antigens, like that of the immunoglobulins, resides in the rearrangement of genes encoding specific Ti subunits termed variable (V), diversity (D), joining (J), and constant (C).^{6,7} The Ti heterodimer is physically linked to the T3 molecule to form a functional Ti-T3 complex.^{4,5,8-10}

The development of molecular probes for the Ti α - and β -chain genes has made it possible to examine the sequence of gene activation that accompanies production of a functional T cell receptor.¹¹⁻¹³ Previously others have used immunoglobulin gene probes and MoAbs to lineage-associated surface antigens to dissect B cell differentiation.¹⁴ We rea-

soned that similar analysis of Ti gene rearrangement and transcription with T cell surface antigens, such as T3 and T11, might yield additional insight into the sequence and regulation of T cell differentiation. The correlations identified here for T lymphoblasts are similar to those for normal thymocytes and indicate that T cells contain cytoplasmic T3 antigen and Ti β -chains transcripts very early in ontogeny. Ti α -chain transcripts and presumably the α -chain protein were present only in blast cells with surface T3 antigen, indicating that α -chain transcription is a critical regulatory event in the formation of the Ti-T3 complex. The presence of β -chain messenger RNA (mRNA) in the absence of α -chain mRNA appears to characterize a stage of T cell differentiation analogous to the pre-B stage of B lymphocytes where heavy-chain Ig genes are expressed but light-chain Ig genes are not.

MATERIALS AND METHODS

Patients. From 10⁷ to 10⁸ marrow blast cells (density <1.077 g/cc) were obtained from five children with untreated T cell acute lymphoblastic leukemia (T-ALL). These cases were selected for analysis because they represented the known stages of T cell differentiation as defined by reactivity with MoAbs. Thymocytes were obtained from normal human thymus removed during corrective cardiac surgery. All studies described in this report were performed with approval of the Clinical Trials Committee of St. Jude Children's Research Hospital and with the informed consent of the patients and their parents.

Immunologic studies. Leukemic blast cells from marrow aspirates were studied for expression of cell-surface antigens by a standard indirect immunofluorescence assay that relied on MoAbs in the cluster groups (CD) known to react with T cell-associated antigens¹⁵ (OKT series, Ortho Diagnostics, Raritan, NJ). Isotypic myeloma immunoglobulins (Littion-Bionetics, Charleston, SC), at a concentration equal to the least diluted MoAb, were used as negative controls; samples demonstrating >10% fluorescence with the myeloma immunoglobulins were considered unacceptable.¹⁶

Cryopreserved leukemic cells and normal thymocytes were tested for cytoplasmic antigens by immunoperoxidase staining.¹⁷ Cytoentrifuged cells (1 to 2 × 10⁵ per slide) were fixed in cold acetone (4 °C for three minutes) and were then stained with either Leu-4 (CD-3) or Leu-5b (CD-2) antibody (Becton Dickinson, Mountain View, CA) at a 1:10 dilution (25 °C for 45 minutes) or isotype-matched

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myeloma immunoglobulins (negative controls). Leu-4 and Leu-5b were used in these experiments because they are superior to OKT3 and OKT11 in the immunoperoxidase assay. Sequentially, with washing in phosphate-buffered saline (PBS) between steps, an affinity-purified biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, CA) was added (25 °C for 20 minutes), followed by avidin DH: biotinylated horseradish peroxidase H complex (Vector Laboratories) (25 °C for 15 minutes) and, finally, 0.1% diaminobenzidine tetrahydrochloride (DAB; Polysciences, Warrington, PA).¹⁸

Cryopreserved leukemic cells were also analyzed for the presence of cytoplasmic antigens by flow cytometry.¹⁹ Briefly, 5×10^6 cells/mL were treated with lysolecithin (lysophosphatidylcholine; Sigma, St Louis) at a concentration of 5 µg/mL in PBS, pH 7.2, without calcium or magnesium (4 °C for three minutes) to permeabilize cell membranes. Washed cells were stained by a standard immunofluorescence technique using a twofold excess of either Leu-5b, (CD-2), Leu-4 (CD-3) antibody or isotype-matched immunoglobulins and were analyzed with an EPICS flow cytometer with a five-W coherent laser (Coulter, Hialeah, FL).

DNA analysis. High-mol wt DNA was prepared from bone marrow cells as described previously, digested with *Bam*HI, *Eco*RI, or *Xba*I restriction endonuclease, electrophoresed through a 0.8% agarose gel, and transferred to nitrocellulose.²⁰ Blots were probed with nick-translated DNA, with plasmid pB400 (provided by Dr M.J. Owen) used as the source of Ti β -chain DNA.²¹ This probe contains 464 nucleotides of the constant region of $C\beta_1$, and 15 bases of the joining region; it hybridizes well to both the Ti β -chain genes. Filters were washed and exposed to Kodak XAR film in the presence of Dupont Lighting Plus intensifying screens.

Northern hybridization analysis. The cDNA clones containing inserts encoding the human Ti α - and β -chain genes have been described.^{21,22} Inserts containing α - and β -genes were isolated and oligolabeled to specific activities of 7 to 20×10^8 cpm/µg of DNA.²³ Total cellular RNA was prepared from leukemic blast cells or the T cell line HPB-ALL by a guanidinium/cesium chloride procedure.²⁴ RNA was denatured in 50% formamide/2.2 mol/L formaldehyde, electrophoresed in 1% agarose, and transferred to nitrocellulose filters. After hybridization for 24 hours, filters were washed in 0.1 mol saline citrate solution at 50 °C (two washed for 15 minutes).

RESULTS

Characteristics of the patients. The clinical characteristics of these four boys and one girl (Table 1) were entirely consistent with ALL of thymic origin. Leukocyte counts exceeded $100 \times 10^9/L$ in all children with a median of $222 \times 10^9/L$ (range, 111.6 to $651.2 \times 10^9/L$). Four patients had a mediastinal mass. Immunologic evaluation (Table 1) established the T cell origin of the cases, with blast cells from all patients reacting strongly with MoAbs that recognize T11 (CD-2) (median 88%, range 85% to 95%). The MoAb binding patterns indicated that the lymphoblasts in case 1 corresponded with stage I thymocytes, those in cases 2 and 3 corresponded with stage II thymocytes, and those in cases 4 and 5 corresponded with stage III thymocytes (Table 1). Surface T3, by definition, was present only on blasts from stage III patients (cases 4 and 5); however, cryopreserved lymphoblasts from all five patients contained large amounts of T3 within the cytoplasm when examined by immunoperoxidase staining (Table 1 and Fig 1A). Furthermore, a higher percentage of cells from patients 4 and 5 contained cytoplasmic T3 by immunoperoxidase staining than was

Table 1. Clinical Characteristics and Immunologic Findings*

	Patient Number				
	1	2	3	4	5
Age (yr)	6.5	12.1	10.8	8.4	15.3
Sex	F	M	M	M	M
Mediastinal mass	Yes	Yes	Yes	No	Yes
Leukocyte count ($\times 10^9/L$)	651.2	201.6	111.6	479.0	222.0
Hemoglobin (g/dL)	7.8	10.9	7.8	7.0	16.0
Platelet count ($\times 10^9/L$)	17.0	64.0	93.0	29.0	16.0
Surface antigens					
T11 (CD-2)	85	95	88	90	86
T6 (CD-1)	24	51	64	15	14
T4 (CD-4)	4	87	9	17	17
T8 (CD-8)	0	86	10	0	8
T3 (CD-3)	1	7	0	48	69
Cytoplasmic antigens†					
T3 (CD-3)	90	90	100	100	98

*All immunologic data are given as percent positive cells.

†Performed by immunoperoxidase staining.

evident from surface immunofluorescence testing (Table 1). When normal thymocytes were studied by immunoperoxidase staining, greater than 90% contained cytoplasmic T3 (Fig 1B).

Flow cytometric analysis of cryopreserved lymphoblasts from patient 2, stained by a standard immunofluorescence technique, failed to disclose surface T3; however, when cell membranes were disrupted with lysolecithin and then stained, 38% of the blasts became positive (Fig 2). The lysolecithin treatment did not change the reactivity pattern seen with the myeloma immunoglobulin negative control or that seen with antibodies T11 and Leu-5 (CD-2).

Molecular analysis. Rearrangement of the two genes encoding the Ti β -chain can be analyzed as a unit by *Bam*HI digestion or separately by *Eco*RI ($C\beta_1$) and *Xba*I ($C\beta_2$) digestions. Table 2 summarizes the results of our analysis of the Ti β -chain gene in DNA from each of the five patients. *Bam*HI digestion demonstrated rearranged Ti β -chain genes in blasts from four of our patients (cases 2 through 5). Examination of the DNA from patient 1 by *Eco*RI and *Xba*I digestions revealed that one $C\beta_1$ gene was rearranged and one was deleted, while one $C\beta_2$ gene was rearranged and one remained in the germ-line configuration (Table 2). Blast cells from patient 2 showed deletion of both $C\beta_1$ genes and rearrangement of both $C\beta_2$ genes. In patient 3 the rearrangements involved only one allele of the $C\beta_1$ gene, and in patient 5 there was deletion of the $C\beta_1$ gene and rearrangement of one of the $C\beta_2$ genes. Patient 4 had an unexpected pattern of β -gene rearrangement. The *Bam*HI digestion pattern indicated that both gene clusters had rearrangements. However, analyses with *Xba*I and *Eco*RI indicated that both rearrangements were in the $C\beta_2$ genes with persistence of the $C\beta_1$ genes in an apparent germ-line configuration. One possible explanation for this finding is the presence of a V region cluster downstream of the $C\beta_2$ constant region.²⁵

Total cytoplasmic RNA was analyzed by the Northern blot technique to determine if the rearranged Ti β -chain genes were being transcribed (Fig 3, Table 2). RNA from patients 1 through 3 and 5 (sufficient cells were not available

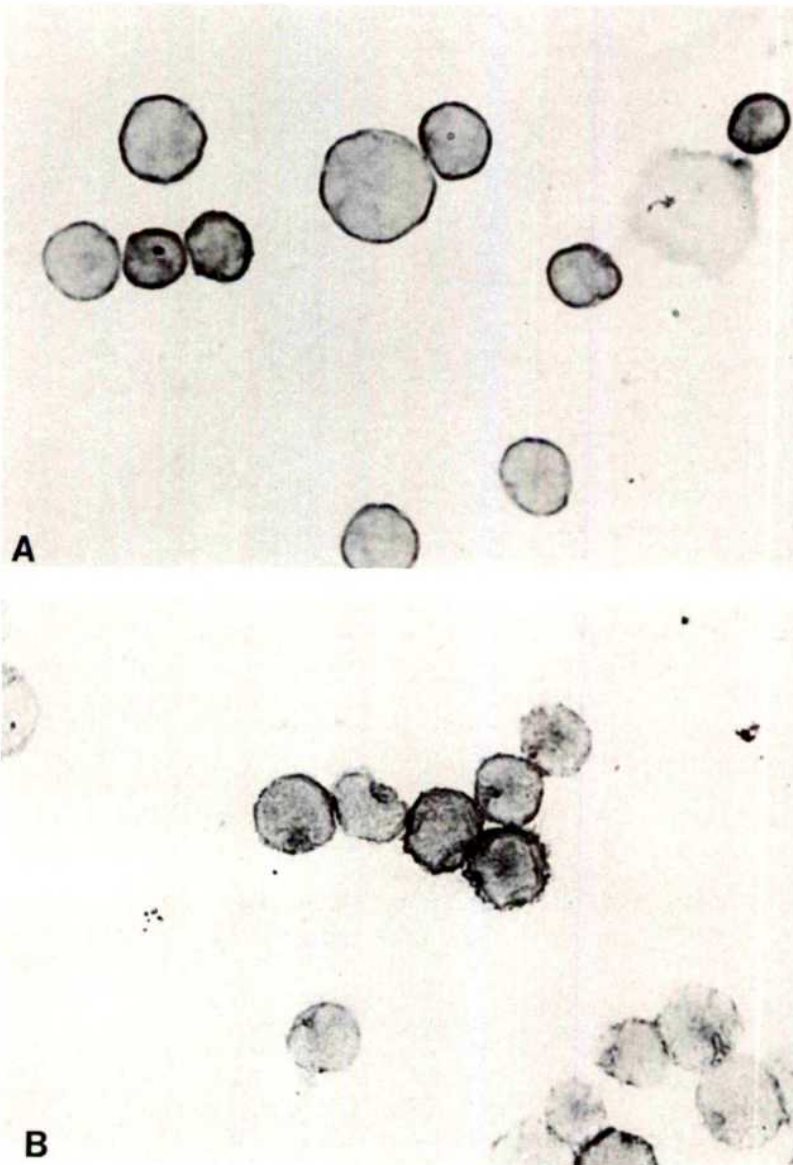


Fig 1. (A) Reactivity of Leu-4 (CD-3) antibody with leukemic blast cells from patient 2, detected by the immunoperoxidase staining technique. (B) Reactivity of Leu-4 antibody with normal thymocytes detected by the immunoperoxidase staining technique.

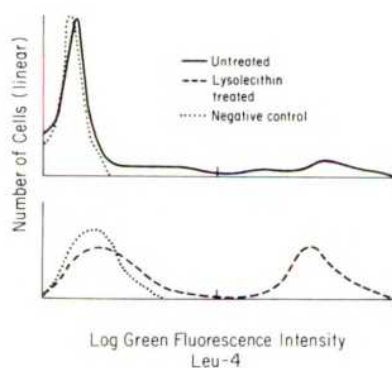


Fig 2. Fluorescence histograms of leukemic cells stained with Leu-4 (CD-3) antibody before (—) and after (---) disruption with lysolcithin. A population of cells was stained by the antibody after treatment. Negative control antibody binding (.....) was not affected by treatment.

from patient 4) contained a 1.3-kilobase (kb) transcript that hybridized to the probe for the β -chain gene (Fig 3). The size of this transcript is similar to that in HPB-ALL cells (stage III, surface T3 positive) producing the protein; hence, the Ti β -chain was probably being synthesized in the blast cells of our patients (data not shown for patient 2). When analyzed with the α -chain probe, total RNA from patients 1 through 3 (surface T3 negative but cytoplasmic T3 positive) lacked transcripts of the α -chain gene. By contrast, RNA from patient 5 (surface T3 positive) included a 1.4-kb band that hybridized to the probe (Fig 3). The similarity of this 1.4-kb band to mRNA transcripts in HPB-ALL cells (surface T3 positive) suggests that functional Ti α -chain was being synthesized. Thus lymphoblasts from all of the patients we studied contained RNA that hybridized to the Ti β -chain gene probe, but only lymphoblasts from patient 5 (surface T3 positive) contained RNA that hybridized to the Ti α -chain probe.

Table 2. Relationship of Molecular Findings to Surface and Cytoplasmic Antigen Expression

	Patient No.				
	1	2	3	4	5
Surface antigens*					
T11	+	+	+	+	+
T6	-	+	+	-	-
T4	-	+	-	-	-
T8	-	+	-	-	-
T3	-	-	-	+	+
Cytoplasmic antigen*					
T3	+	+	+	+	+
Ti β-chain genes					
C β_1	R/D	D/D	R/G	G/G	D/G
C β_2	R/G	R/R	G/G	R/R	R/G
RNA transcripts					
β -Chain gene	Yes	Yes	Yes	ND	Yes
α -Chain gene	No	No	No	ND	Yes

Abbreviations: R, rearranged; G, germline, D, deleted, ND, not determined.

*A minus sign indicates <35% positive cells, a plus sign 35% or greater positive cells.

DISCUSSION

Our results demonstrate discordance between the surface and cytoplasmic expression of T3 antigen in leukemic cells. Cytoplasmic presence of T3 antigen was detected in all MoAb-defined stages of development, but surface T3 was present only in stage III. Link et al¹⁸ have also described cytoplasmic T3 expression without surface T3 expression in 16 of 17 cases of T cell ALL and T cell lymphoblastic lymphoma. Additionally we found that greater than 90% of the normal thymic cells contain cytoplasmic T3, whereas a lower percentage of these cells show surface T3. The results with normal thymocytes strongly suggest that cytoplasmic T3 positive, surface T3 negative leukemic cells correspond to a normal stage in T cell differentiation.

We also examined the relationship of Ti gene rearrangement and/or their transcription to cytoplasmic and surface T3 expression in T cell ontogeny. Similar to other investigators,^{2,6,7,26} we found Ti β -chain gene rearrangements in leukemic cells representing all three stages of thymocyte development. We could not examine DNA for rearrangements of Ti α -chain genes, as probes suitable for completely examining the full length of this estimated 50-kb gene are not yet available.²⁷ Lacking a pan-Ti antibody, we examined total RNA from four cases for the presence of α - and β -chain transcripts in an effort to clarify the differential expression of genes encoding the T cell receptor in lymphoblasts. This analysis indicated that transcripts of the Ti β -chain gene are present even in the earliest stage of T cell differentiation detectable by MoAbs. In patients with blasts containing cytoplasmic T3 but not surface T3, we could not demonstrate Ti α -chain gene transcripts. However, in surface T3-positive lymphoblasts, both β - and α -chain transcripts were present. A similarly ordered transcription of α and β Ti genes has been seen during intrathymic ontogeny.^{28,29} The findings we report expand our understanding of thymocyte development. T cells containing Ti β mRNA and presumably expressing

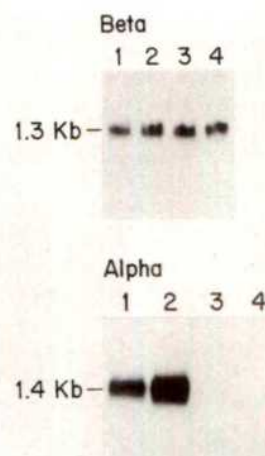


Fig 3. Northern blot analysis of total cellular RNA to identify transcripts of Ti β - and α -chain genes. β -Chain gene: lane 1 contains control RNA from HPB-ALL cells (surface T3⁺), lane 2 contains RNA from patient 1 (cytoplasmic T3⁺, surface T3⁻), lane 3 contains RNA from patient 3 (cytoplasmic T3⁺, surface T3⁻), and lane 4 contains RNA from patient 5 (surface T3⁺). α -Chain gene: lane 1 contains RNA from patient 5, lane 2 contains control RNA from HPB-ALL cells, lane 3 contains RNA from patient 1, and lane 4 contains RNA from patient 3. No α -chain gene hybridizing bands are evident in lanes 3 and 4 (patients 1 and 3).

the β -chain protein but lacking Ti α mRNA and protein would appear to be analogous to pre-B cells where the heavy chain Ig gene protein but not the light-chain Ig gene protein is expressed.³⁰ These pre-T cells would include both stage I and II thymocytes in the Reinherz et al schema.¹

Taken together, our results on the cytoplasmic and surface expression of T3 and transcription of α and β Ti genes suggest that transcription of the α -chain gene is a critical regulatory event in Ti formation and transport of the T3 antigen to the cell surface. It is possible that in lymphoblasts the Ti α -chain genes are rearranged, but no mRNA is produced because of nonproductive somatic rearrangements, similar to the situation with immunoglobulin genes. The differentiation sequence we have identified in leukemia appears identical to that occurring in normal thymocytes.^{28,29}

Leukemic blast cells from all patients and most thymocytes expressed surface T11 (CD-2), contained rearranged and transcribed Ti β -chain genes, and had cytoplasmic T3. Since this manuscript was submitted, results similar to these have been reported by Furley et al, and these investigators have identified thymocytes with cytoplasmic T3 without Ti β -chain rearrangement.³¹ Taken together, our results and those of Furley et al indicate that the hierarchy of T cell gene rearrangements and expression (T11, T3, Ti, and others) is much more complex than first thought and will require further combined immunologic and molecular studies with thymocytes and leukemic T cells to be fully elucidated.

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The Influence In Vivo of Murine Colony-Stimulating Factor-1 on Myeloid Progenitor Cells in Mice Recovering From Sublethal Dosages of Cyclophosphamide

By Hal E. Broxmeyer, Douglas E. Williams, Scott Cooper, Abdul Waheed, and Richard K. Shadduck

Pure murine colony-stimulating factor-1 (CSF-1) was assessed for its effects in vivo in mice pretreated seven days earlier with a sublethal dosage of cyclophosphamide. The multipotential (CFU-GEMM), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) progenitor cells in these mice were in a slowly cycling or noncycling state. Intravenous administration of 20,000 units of CSF-1 to these mice stimulated the hematopoietic progenitors into a rapidly cycling state in the marrow and spleen within three hours. Significant increases in absolute numbers of marrow and spleen CFU-GM and spleen BFU-E and CFU-GEMM were also detected. No endotoxin was detected in the CSF-1 preparation by *Limulus* lysate assay, and treatment

of CSF-1 at 100 °C for 20 to 30 minutes completely inactivated the in vitro and in vivo stimulating effects. The effects of CSF-1 were not mimicked by the in vivo administration of 0.1 to 10 ng *Escherichia coli* lipopolysaccharide. These results suggest that the effects of CSF-1 in vivo were not due to contaminating endotoxin or to a nonspecific protein effect. CSF-1 did not enhance colony formation by BFU-E or stimulate colony formation by CFU-GEMM in vitro, thus suggesting that at least some of the effects of CSF-1 noted in vivo are probably indirect and mediated by accessory cells.

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COLONY-STIMULATING FACTOR-1 (CSF-1), macrophage CSF, is one of a group of CSFs defined operationally by their ability to stimulate colony formation by hematopoietic progenitor cells in semisolid culture medium,^{1,2} although it is apparent that CSFs can also influence the functional capacity of more mature cells.³⁻⁵ CSF-1 from human and murine sources have been purified to homogeneity,⁶⁻⁹ and the complementary DNA for CSF-1 derived from a human cell line has been cloned and expressed.¹⁰ Information is available on the production,¹¹ receptor-binding capability,¹²⁻¹⁹ and actions in vitro of CSF-1,³⁻⁵ but there is a paucity of information on the effects of CSF-1 in vivo²⁰⁻²² and evidence that CSF-1 may function in vivo is mainly indirect.

The present studies were designed to evaluate the short-term effects in vivo of a single injection of endotoxin-free purified murine CSF-1 on myelopoiesis in mice pretreated with a sublethal dosage of cyclophosphamide. It was reasoned that it might be easier to detect an effect of exogenously administered CSF-1 in mice in which the proliferative status of hematopoietic progenitor cells was suppressed during the overbound phase of myelopoiesis resulting from the cytotoxic effects of cyclophosphamide. This model allowed the detection of a significant proliferative effect of CSF-1 in vivo on bone marrow granulocyte-macrophage (CFU-GM) and macrophage (CFU-M) progenitor cells and on spleen CFU-GM, multipotential (CFU-GEMM), and erythroid (BFU-E) progenitor cells.

MATERIALS AND METHODS

Mice. (C57B1/6 × DBA/2) F₁ (BDF₁) mice, 6 to 8 weeks old, were purchased from Cumberland View Farms (Clinton, TN).

Molecules. L cell CSF (CSF-1) was produced by the growth of murine L cells in serum-free CMRL 1066 medium.⁷ A 10-L pool of conditioned medium was purified by affinity chromatography⁸ and had a specific activity of 2.3×10^7 U/mg of protein when assayed by the in vitro growth of murine bone marrow colonies. This material was subjected to sucrose gradient centrifugation as a means of removing any potential endotoxin.²³ After centrifugation at 100,000 g for 18 hours, the upper third of the sucrose gradient was aspirated and diluted in 0.1 mmol/L Tris-HCL containing 0.3% polyethylene glycol 4000 as a stabilizing agent. A control solution was prepared

using the upper third of a sucrose gradient that did not contain CSF. The CSF-1 was injected in a volume of 0.2 mL and had no detectable endotoxin as determined by the *Limulus* lysate assay (Sigma Chemical Co, St Louis), which can detect endotoxin concentrations down to 0.5 ng/mL. Cyclophosphamide (Cytosan, Bristol Myers Oncology Division, Syracuse, NY) was diluted in sterile pyrogen-free water prior to intraperitoneal (IP) injection.

Cell preparation. Both femurs were excised and the shafts cleaned of residual muscle tissue. The ends of each bone were then carefully cut off without exposing the marrow cavity. To wash cells out of the femurs, a sterile 27-gauge needle was forced into the upper and lower end of the femur to allow fluid to pass through the bone. Cells were then flushed from each femur by passing 5 mL of ice cold McCoy's 5A medium (GIBCO, Grand Island, NY) through each bone. Medium was forced through both ends of each bone to ensure that the maximum number of cells was removed from each bone shaft. Cells from two femurs per animal were pooled and single-cell suspensions made by passage through a 23-gauge needle. Spleens were removed and placed in 60 × 15-mm tissue culture dishes containing 10 mL of McCoy's 5A medium. Spleens were forced through a sterile wire mesh screen several times using the sterile barrel of a 10 mL syringe. Single-cell suspensions were made as for marrow cells by passing cells through a 23-gauge needle. The viability of suspended marrow and spleen cells was routinely >98%.

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Table 1. Influence of Purified Murine CSF-1 In Vivo on Cellularity, Hematopoietic Progenitor Cells, and the Cycling Status of Progenitor Cells in the Marrow and Spleen

Test Material	Nucleated Cells $\times 10^{-6}$ per Organ	Colonies $\times 10^{-3}$ /Organ (% Δ)*			Percentage of Cells in S-Phase				
		CFU-GM	BFU-E-2	BFU-E-1	CFU-GEMM	CFU-GM	BFU-E-2	BFU-E-1	CFU-GEMM
Bone Marrow									
Control diluent	17 $\pm$ 1	2.9 $\pm$ 0.5	NS†	NS	NS	16 $\pm$ 4	NS	NS	NS
CSF-1 (20,000 units)	19 $\pm$ 1 (+12)	5.5 $\pm$ 1.0 (+90)†	NS	NS	NS	59 $\pm$ 4†	NS	NS	NS
Heat-treated CSF-1	19 $\pm$ 1 (+12)	2.7 $\pm$ 0.5 (-7)	NS	NS	NS	4 $\pm$ 3	NS	NS	NS
LPS (10 ng)	16 $\pm$ 1 (-6)	1.8 $\pm$ 0.5 (-38)	NS	NS	NS	-2 $\pm$ 2§	NS	NS	NS
LPS (1 ng)	19 $\pm$ 1 (+12)	2.1 $\pm$ 0.4 (-28)	NS	NS	NS	2 $\pm$ 4§	NS	NS	NS
LPS (0.1 ng)	18 $\pm$ 1 (+6)	2.5 $\pm$ 0.1 (-14)	NS	NS	NS	2 $\pm$ 2§	NS	NS	NS
Spleen									
Control diluent	58 $\pm$ 5	188 $\pm$ 20	52 $\pm$ 9	41 $\pm$ 8	36 $\pm$ 4	3 $\pm$ 2	6 $\pm$ 5	1 $\pm$ 5	-5 $\pm$ 4
CSF-1 (20,000 units)	61 $\pm$ 5 (+5)	254 $\pm$ 25 (+35)†	95 $\pm$ 11 (+83)†	44 $\pm$ 5 (+7)	56 $\pm$ 8 (+55)†	50 $\pm$ 3†	57 $\pm$ 4†	54 $\pm$ 3†	62 $\pm$ 5†
Heat-treated CSF-1	56 $\pm$ 3 (-3)	226 $\pm$ 21 (+20)	59 $\pm$ 6 (+13)	44 $\pm$ 6 (+7)	34 $\pm$ 7 (-6)	2 $\pm$ 3	4 $\pm$ 6	-1 $\pm$ 4	-8 $\pm$ 3
LPS (10 ng)	59 $\pm$ 4 (+2)	224 $\pm$ 19 (+19)	57 $\pm$ 4 (+10)	46 $\pm$ 2 (+12)	21 $\pm$ 2 (-42)§	1 $\pm$ 2	-8 $\pm$ 2	-2 $\pm$ 1	-9 $\pm$ 4
LPS (1 ng)	63 $\pm$ 8 (+9)	222 $\pm$ 33 (+18)	56 $\pm$ 4 (+8)	43 $\pm$ 3 (+5)	23 $\pm$ 5 (-36)§	2 $\pm$ 2	-2 $\pm$ 4	6 $\pm$ 3	4 $\pm$ 4
LPS (0.1 ng)	57 $\pm$ 6 (-2)	225 $\pm$ 19 (+20)	53 $\pm$ 8 (+2)	48 $\pm$ 7 (+17)	20 $\pm$ 3 (-44)§	1 $\pm$ 3	0 $\pm$ 3	-3 $\pm$ 3	-5 $\pm$ 4

Mice were injected IP with 200 mg cyclophosphamide/kg body weight. Seven days later the mice were injected IV with control diluent, 20,000 units purified CSF-1, 20,000 units of CSF-1 that had been heated at 100 °C for 20 to 30 minutes, or 10, 1, or 0.1 ng *E. coli*/lipopolysaccharide (LPS). The mice were sacrificed three hours later. For control diluent or CSF-1, results are the averages of 15 mice each (in a total of five experiments). For heat-treated CSF-1, results are the averages of 11 mice (in a total of four experiments), and for LPS the results are the averages of six mice each (in a total of two experiments).

*The percent change from control diluent (% Δ) is given in parenthesis.

†NS, not scored. Control colony numbers were too low to use.

‡Significant percent increase from control diluent, P at least $< .05$.

§Significant percent decrease from control diluent, P at least $< .05$.

Analysis of hematopoietic progenitor cells in vitro. The assay for CFU-GM was performed as described²⁴ by using pokeweed mitogen mouse spleen cell conditioned medium (PWMSCM) as a source of CSF.²⁴ Bone marrow and spleen cells from cyclophosphamide-treated mice were plated respectively at 2×10^5 and 2.5×10^4 cells/mL in 0.3% agar culture medium (Difco Laboratories, Detroit) that included McCoy's 5A medium supplemented with essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate (GIBCO) with 10% prescreened heat-inactivated (56 °C for one-half hour) fetal bovine serum (Hyclone, Logan, UT). Colonies (>50 cells/aggregate) were scored after seven days of incubation. For colony morphology, agar cultures were overlaid with 1 mL of 0.5% glutaraldehyde (Sigma) in 0.1 mol/L phosphate buffer. After six minutes, the glutaraldehyde was removed, and 2 mL absolute methanol was added to the plates. After the methanol evaporated (12 to 18 hours) the fixed cultures were submerged in distilled water. After rehydration, the agar pellicles were floated onto clean glass slides and allowed to dry. For identification of colonies containing granulocytes and monocytes/macrophages, the colonies were stained for 45 minutes with α -naphthyl acetate esterase (Sigma Kit #90-A1) and counterstained with Mayer's hematoxylin for ten minutes. The slides were washed, dried at room temperature, mounted (Permount, Fisher, St Louis) and scored at 400 $\times$. Monocytes/macrophages were enumerated based on their morphology and the characteristic black cytoplasmic granulation. Granulocytes were nonspecific esterase-negative and had characteristic nuclear morphology.

The assay for BFU-E-2 was plated at 1×10^5 cells/mL for marrow and 2.5×10^4 cells/mL for spleen from cyclophosphamide-treated mice in a 1-mL mixture of Iscove's modified Dulbecco's medium (GIBCO), 0.8% methylcellulose, 30% prescreened fetal bovine serum (Hyclone), 5×10^{-5} mol/L 2-mercaptoethanol, 1 unit of erythropoietin (Hyclone), and 0.1 mmol/L hemin (Eastman Kodak, Rochester, NY). Hemin increases colony formation by BFU-E and CFU-GEMM.²⁵ BFU-E-1 and CFU-GEMM assays were set up as for BFU-E-2 except that 1% vol/vol PWMSCM was added and colonies were scored from the same plates. Cells were plated at 1.0×10^5 cells/mL for bone marrow and 5×10^3 to 2.5×10^4 cells/mL for spleen BFU-E-1 and CFU-GEMM assays. BFU-E-2, BFU-E-1, and CFU-GEMM assays were scored after six to seven days of incubation. The terms BFU-E-2 and BFU-E-1 are operational only because the colonies derived from BFU-E-1 were much larger than those derived from BFU-E-2. It is possible that BFU-E-1 may represent a more immature progenitor than BFU-E-2, although this has not been proved.

Cells were incubated in a humidified environment at lowered (5%) oxygen tension. Low oxygen tension was maintained using an Oxyreducer (Reming Bioinstruments, Rochester, NY) since lowered oxygen tension increases the incidence of colonies derived from hematopoietic progenitor cells.²⁶

Cycle status of hematopoietic progenitor cells. To estimate the proportion of progenitors in DNA synthesis (S-phase), cells were treated with either McCoy's medium or 50 μ Ci tritiated thymidine (³HTdR, New England Nuclear, Boston; specific activity, 20 Ci/mmol). After a 20-minute incubation at 37 °C, the cells were washed once in a buffer containing ice cold thymidine (2,000 μ g), twice more in McCoy's medium, and then plated into the CFU-GM, BFU-E, and CFU-GEMM assays. The reduction in the number of colonies after exposure of the cells to ³HTdR compared with the control (McCoy's medium) estimates the proportion of progenitor cells in cycle just prior to the assay for colony formation. The results are given as the percentage of cells in S-phase. A negative number means that more colonies formed after the cells were exposed to ³HTdR than after the cells were exposed to McCoy's medium. In some select cases, cells were also exposed to 500 μ g thymidine or 50

μ Ci ³HTdR plus 500 μ g thymidine as controls for the S-phase specificity of the treatments. In all cases, the numbers of colonies formed were statistically similar whether the cells were exposed to McCoy's medium, thymidine, or ³HTdR plus thymidine. The percentage of cells in S-phase was based on control colony counts from cells pulsed with McCoy's medium that ranged from 50 to 185, 18 to 37, 15 to 41, and 14 to 45, respectively, for the CFU-GM, BFU-E-2, BFU-E-1, and CFU-GEMM assays.

Experimental protocols. The details of these are described in Results and in the tables. The numbers of mice used are given in the table legends. After sacrifice, the marrow from the two femurs of each mouse was pooled. This marrow and the spleen of each mouse were assayed separately. Cells from different mice were not pooled.

Statistical analysis. Three plates were scored for each CFU-GM sample, and two plates were scored for each BFU-E-2 or BFU-E-1/CFU-GEMM sample. The results are expressed as the mean $\pm$ 1 SEM, and these are derived from the averages of the colony counts from each of the individual mice within a group. The probability of significant differences between groups or samples was determined with the use of Student's *t* test.

RESULTS

Influence of CSF-1 and Escherichia coli lipopolysaccharide in mice pretreated with cyclophosphamide. In preliminary experiments we noted that the cycling status of myeloid progenitor cells was significantly decreased in the bone marrows and spleens removed from mice seven days after IP administration of cyclophosphamide (200 mg/kg body weight) when compared with cells from untreated mice (data not shown). Cyclophosphamide-pretreated mice were thus used to evaluate the potential proliferative effects of CSF-1 in vivo on myeloid progenitors. The mice were injected IP with 200 mg cyclophosphamide followed by a second intravenous (IV) injection seven days later with control diluent, 20,000 units CSF-1, 20,000 units CSF-1 that had been treated at 100 °C for 20 to 30 minutes, or with 0.1 to 10 ng *E. coli* lipopolysaccharide and sacrificed three hours later. The results for the averages of two to five separate experiments are shown in Table 1. In five experiments, CSF-1 had no significant effect on nucleated marrow or spleen cells but significantly increased the absolute numbers of marrow CFU-GM and splenic CFU-GM, BFU-E-2, and CFU-GEMM. CSF-1 also significantly increased the percentage of marrow CFU-GM and splenic CFU-GM, BFU-E-2, BFU-E-1, and CFU-GEMM in cycle. The numbers of marrow BFU-E and CFU-GEMM were too low to evaluate. The increase in cycling CFU-GM was apparent for granulocyte and granulocyte-macrophage progenitors as well as for macrophage progenitors (Table 2). Control diluent had no effects on any of the parameters evaluated that were significantly different from that of sterile pyrogen-free saline (data not shown).

Heat-treated CSF-1 and *E. coli* lipopolysaccharide were used as controls. The effect of heating CSF-1 in vitro at 100 °C for 5, 10, and 20 minutes was assessed on the activity of CSF-1 in vitro. Fifty units of untreated CSF-1 stimulated 101 ± 7 colonies and 203 ± 17 colonies plus clusters per 7.5×10^4 bone marrow cells from untreated mice. After heat treatment of CSF-1, colony, and colony plus cluster, the numbers were reduced to 6 ± 1 and 44 ± 1 , 0 and 2 ± 1 , and 0

Table 2. Influence of Purified CSF-1 In Vivo on Cycling Status of Granulocyte-Macrophage Progenitor Cells

Animal	Treatment In Vitro With	Colonies per No. of Cells Plated (%Δ)	Colony Morphology (%)		
			G	M	GM
Bone Marrow					
No. 1	Control medium	57 ± 5	2	65	33
	³ HTdr	24 ± 3 (–58)*	3	64	33
No. 2	Control medium	48 ± 2	5	61	34
	³ HTdr	16 ± 1 (–67)*	1	66	33
No. 3	Control medium	82 ± 3	1	68	31
	³ HTdr	20 ± 2 (–76)*	1	65	34
Spleen					
No. 1	Control medium	85 ± 1	8	60	32
	³ HTdr	47 ± 4 (–45)*	17	52	31
No. 2	Control medium	79 ± 3	9	68	23
	³ HTdr	45 ± 2 (–43)*	6	60	34
No. 3	Control medium	109 ± 3	5	74	21
	³ HTdr	58 ± 5 (–47)*	8	69	23

The mice were injected IP with 200 mg Cytoxan/kg. Seven days later the mice were injected IV with 20,000 units purified CSF-1, and the mice were sacrificed three hours later. Results shown are for three mice each receiving CSF-1 in vivo. The numbers in parentheses designate the percent change from control (%Δ).

Abbreviations: G, granulocyte; M, macrophage; GM, granulocyte-macrophage.

*Significant percent change from control, *P* at least <.05.

and 0 respectively for preparations of CSF-1 heat-treated for 5, 10, and 20 minutes. In another experiment, CSF-1 stimulated 33 ± 2 colonies and 114 ± 7 colonies plus clusters in vitro, whereas CSF-1 heat-treated at 100 °C for 30 minutes stimulated no colonies and 1.3 ± 0.7 clusters. In each of these experiments the background growth in the absence of CSF-1 was <2 small clusters. Based on this information, CSF-1 was treated at 100 °C for 20 minutes (two experiments) or for 30 minutes (two experiments) and compared with untreated CSF-1 for its effects in vivo (Table 1). Heat-treating the CSF-1 inactivated its effects in vivo. The administration of 0.1, 1.0, or 10 ng *E coli* lipopolysaccharide to mice in vivo (Table 1) had no significant enhancing effect on myelopoiesis and in some cases resulted in significant decreases.

In two experiments the marrow and splenic morphology was evaluated. The percentage of marrow cells from mice treated with control diluent was 5% to 8% myeloblasts and promyelocytes, 66.5% to 73% myelocytes, 12.5% to 13.5% metamyelocytes and bands, 0.5% to 1.5% polymorphonuclear cells (PMN), 4% to 5% monocytes, 2.5% to 4%

lymphocytes, and 0.5% to 2.5% nucleated erythroid cells. The percentage of splenic cells from mice treated with control diluent was 7% to 8.5% myeloblasts and promyelocytes, 29% to 31% myelocytes, 19% to 21.5% metamyelocytes and bands, 9.5% to 12.5% PMN, 2.5% to 4% monocytes, 18% to 21% lymphocytes, and 5.5% to 10% nucleated erythroid cells. These differentials were not significantly changed by administration to the cyclophosphamide-pre-treated mice of CSF-1, heat-treated CSF-1, or 0.1, 1, or 10 ng *E coli* lipopolysaccharide.

Influence of CSF-1 in vitro. The effects of CSF-1 on erythroid and multipotential progenitors was of interest, since we could not detect burst-promoting activity (BPA) or mixed colony (CFU-GEMM) activity in the CSF-1 preparation during assay in vitro. At higher concentrations, CSF-1 decreased colony formation by BFU-E (Table 3).

DISCUSSION

Purified murine CSF-1 that is essentially free of endotoxin (<0.1 ng/injection) has significant stimulating effects on

Table 3. Influence of Pure Murine CSF-1 and PWMSCM on Colony Formation by Murine Marrow BFU-E and CFU-GEMM

Treatment	Colonies					
	Exp 1		Exp 2		Exp 3	
	BFU-E	CFU-GEMM	BFU-E	CFU-GEMM	BFU-E	CFU-GEMM
McCoy's Medium CSF-1	21 ± 1	0.5 ± 0.5	12 ± 2	2 ± 1	7 ± 2	0
200 units	10 ± 0*	0	—	—	—	—
100 units	7 ± 1*	0	3 ± 1*	1 ± 0	3 ± 1*	0
50 units	13 ± 4*	0	4 ± 2*	2 ± 1	4 ± 1*	0
25 units	16 ± 1*	0.5 ± 0.5	10 ± 1	2 ± 1	8 ± 2	0
10 units	21 ± 2	0.5 ± 0.5	9 ± 2	1 ± 1	6 ± 1	0
PWMSCM (5% vol/vol)	35 ± 2†	20 ± 1†	21 ± 3†	11 ± 1†	24 ± 2†	15 ± 2†

Bone marrow cells from untreated BDF₁ mice were plated at 7.5×10^4 /mL in the presence of 1 unit erythropoietin and 0.1 mmol/L hemin. Colonies were scored after seven days of incubation.

*Significant decrease from control medium, *P* at least <.05.

†Significant increase from control medium, *P* at least <.05.

marrow and splenic hematopoietic progenitor cells when administered to mice seven days after injection with a sublethal dose of cyclophosphamide. Within three hours of the injection of CSF-1 into mice, the CFU-GEMM, BFU-E, and CFU-GM were induced from a slowly cycling or noncycling state into a rapidly cycling state. Additionally, significant stimulating effects were detected on absolute numbers of marrow and spleen CFU-GM and spleen BFU-E and CFU-GEMM. At this time we did not detect a significant effect on the total nucleated marrow, spleen, or peripheral blood counts or differentials (data not shown). Of interest and potential relevance is the fact that the effects of CSF-1 in vivo were more evident on induction of cycling of progenitors than on changes in progenitor cell numbers. We chose to evaluate the mice three hours after CSF-1 administration because CSF-1 can induce the release in vitro of prostaglandin E,²⁷ acidic isoferritins,⁴ interferon- α ,^{28,29} and tumor necrosis factor²⁹ from monocytes/macrophages and these molecules have suppressive effects in vitro for myeloid progenitor cells. The CSF-1-induced release of these suppressor molecules is usually first detected 12 to 24 hours after the addition of CSF-1 to the cell cultures. It was hoped that by assessing mice within three hours after the administration of CSF-1 in vivo we might obviate the potential CSF-1 induction of suppressor molecules, which might have masked the stimulating effects of CSF-1.

Since endotoxin was not detected in our preparation of CSF-1, at least within the limits of the *Limulus* lysate assay, it does not appear likely that the effects of CSF-1 in vivo are

due to contaminating endotoxin. This belief is further supported by our studies demonstrating that treatment of CSF-1 at 100 °C for 20 to 30 minutes inactivated its stimulating capacity both in vitro and in vivo. This treatment would not have inactivated endotoxin.³⁰ Moreover, the administration of 0.1 to 10 ng *E coli* lipopolysaccharide to these mice did not mimic the effects of CSF-1. The studies with heat-inactivated CSF-1 were also important as controls for the potential nonspecific stimulating effects of proteins in general.

It is not possible to conclude from these studies whether the effects of CSF-1 in vivo are direct ones on the hematopoietic progenitor cells in the marrow and spleen, whether these effects are mediated through an action on accessory cells present in the blood, marrow, or spleen, or whether the actions are both direct and indirect. Although CSF-1 can stimulate colony formation by granulocytes, granulocytes plus macrophages, and macrophages in vitro from an unseparated population of marrow cells,^{31,26} we did not detect BPA or multipotential (CFU-GEMM) activities in vitro with our preparation of CSF-1. CSF-1 can induce the elaboration of other species of CSF from cells in vitro,^{29,32,33} and it is probable that at least some of the effects noted in vivo are mediated through the induced release by CSF-1 of other growth factors for hematopoietic progenitor cells.

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Monoclonal Anti-Light Chain Idiotypic as a Tumor-Specific Probe for Human Neoplastic B Lymphocytes

By M. Wraitham, A.L. Tutt, M.J. Glennie, T.J. Hamblin, G.T. Stevenson, and F.K. Stevenson

Tumor cells from patients with B cell neoplasms often secrete small amounts of free monoclonal light chains that can be found in the urine. Such tumor-derived light chains of the λ type from a patient with typical chronic lymphocytic leukemia have been used to raise mouse monoclonal antibodies (MoAbs). A hybridoma-secreting antibody that recognized the idiotypic λ chain but not normal λ chains by a preliminary screen but which also reacted with idiotypic IgM from the patient's tumor cells was selected. This MoAb in fact recognized 1 in 20×10^3 molecules of pooled normal λ chains, thus establishing its specificity for a private idiotypic determinant. It failed to give a detectable reaction with normal IgM, normal serum, or a panel of IgM paraproteins. The antibody bound to the patient's neoplas-

tic B cells but not to normal tonsillar cells. The site of binding of the antibody to idiotypic IgM is clearly separate from that of another MoAb specific for idiotypic determinants on heavy plus light chains, since the two showed additive binding curves. The determinant also appeared to be less available in dimeric λ chains than in monomeric λ chains or in idiotypic IgM. Antibodies to idiotypic determinants on light chains show some technical advantages and should be useful for monitoring and possibly treating B cell tumors, either alone or together with the more conventional anti-idiotypic antibodies that usually recognize the heavy and light chain combination.

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THE IDIOTYPIC determinants of the surface immunoglobulin (Ig) of neoplastic B lymphocytes represent a molecularly defined tumor-associated antigen.¹ Antibodies specific for these determinants have been used for treatment of human lymphoma with some indication of success, although difficulties remain.^{2,3} Monitoring of disease with anti-idiotypic, however, either by identification of tumor cells or by assay of free serum idiotype, presents few problems.^{4,5}

Nevertheless, difficulty in raising such antibodies has prevented widespread use by clinicians, since idiotypic Ig for immunization must first be obtained from the tumor cells, perhaps from a small biopsy, either by enzyme digestion⁶ or by rescue hybridization,⁷ neither of which is a routine procedure. The use of impure immunogen such as tumor cells or membrane preparations, although possible, yields only a small proportion of anti-idiotypic hybridomas from which to select. The use of an alternative source of immunogen, such as the monoclonal light chain derived from tumor cells and found in urine, therefore could represent a simplification of the approach.

In a study of patients with non-Hodgkin's lymphoma, 23/24 patients had tumor cells that secreted free monotypic light chains.⁵ In a separate investigation of random urine samples from patients with B cell disease, about 50% showed monoclonal light chain by sensitive isoelectric focusing.⁸ Presumably the level of the urinary light chain depends partly on tumor load. Such light chains can be prepared by simple immunosorption methods and have been used previously to raise polyclonal antisera for four patients with chronic lymphocytic leukemia (CLL).⁹ One notable finding was that these four antisera, rendered specific for idiotype by absorption, recognized the cell surface IgM on tumor cells from the original patients. The finding was unexpected, since in the reciprocal situation anti-idiotypic raised against combined (heavy plus light chain) idiotype usually does not recognize free light chain.¹⁰

To analyze more critically whether the same antibody could recognize both free and combined light chain idiotype, we have raised a monoclonal antibody (MoAb) with this dual recognition. We have also compared it with a conventional anti-idiotypic antibody raised against idiotypic IgM from the same patient.

MATERIALS AND METHODS

Patient and Cell Preparation

The male patient LP, aged 50 years, presented with typical CLL with a WBC of $50 \times 10^9/L$ consisting of >95% neoplastic B cells that expressed IgM and IgD, both with the λ light chain, on the surface with no detectable intracellular Ig. A bone marrow sample also showed heavy infiltration with tumor cells, >90% expressing IgMD λ . Serum immunoglobulins were at the low end of the normal range, and no paraprotein was detected on routine electrophoresis. Cells were prepared from peripheral blood and were usually frozen in a controlled cell freezer unit for subsequent investigation.⁹ Urine was collected as 24-hour samples directly into bottles containing 5 mL toluene as preservative.

Urinary Light Chain

A sample of concentrated ($\times 100$) urine was analyzed by isoelectric focusing (IEF) with immunofixation as described.⁸ Preparation of the identified λ chain was by sequential immunosorption.⁹ Briefly, urine (500 mL) was passed through a series of 10-mL columns consisting of Sepharose 4B-CL linked to: (1) normal sheep IgG to remove nonspecific adherent material; (2) sheep anti- κ to remove κ light chains; and (3) sheep anti- λ to bind the λ chains. Elution of the last column with 0.5 mol/L NH_4OH -1.0 mol/L KSCN, followed by dialysis into cold 0.1 mol/L Tris-HCl-1 mol/L NaCl to 0.2% NaN_3 , pH 8, isolated the relevant light chain. Any sheep protein detached from the immunosorbent during elution was removed by passage through an immunosorbent of rabbit antish sheep IgG (5 mL). The purity of the final product was examined by IEF, and amounts were

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measured at each stage by enzyme-linked immunosorbent assay (ELISA). Separation of disulfide-bonded dimeric from monomeric λ chain was by passage through a column of Sephadex G-100 (Pharmacia) equilibrated with propionic acid (1 mol/L). The separated peaks were dialyzed into acetate buffer (4 mmol/L, pH 5.4) and stored at -20°C . To obtain normal λ chains, IgG λ was first prepared from normal human IgG by immunosorption. This IgG λ was then reduced and alkylated and the monomeric λ chains separated in propionic acid as above.

Monoclonal Anti-idiotypes

Anti- λ chain idiotypic (anti-Id [λ]). The isolated urinary λ chain was used to immunize a BALB/c mouse with a primary subcutaneous (SC) injection of 100 μg in Freund's complete adjuvant (CFA), followed by similar booster injections after 5, 7, and 9 weeks. The final injection was of 100 μg aqueous antigen intravenously (IV) three days before removal of the spleen for hybridization of the cells with the mouse myeloma NS-1 (P3-NS-1/1-Ag 4.1).¹¹ The procedures for growing colonies and cloning were as described,¹² selection being based on reactivity of supernatants in the ELISA system with idiotypic λ chain (λId), IgMId, and irrelevant light chains. To produce larger amounts, 10^7 hybridoma cells were injected intraperitoneally (IP) into Pristane-primed BALB/c mice, and antibody was purified from ascitic fluid by precipitation with ammonium sulfate followed by separation on a column of diethyl aminoethyl (DEAE)-trisacryl in phosphate buffer (5 mmol/L, pH 8) with a linear gradient to 50 mmol/L. The major peak was collected and its purity checked by electrophoresis.

Anticombined idiotypic (anti-Id [$\mu + \lambda$]). To obtain IgMId for immunization, a "rescue" hybridization was carried out by fusing tumor cells from the blood of patient LP with the mouse myeloma NS-O, a subline that synthesizes no mouse κ chain. Colonies producing IgM λ were cloned by limiting dilution and supernatants from confluent growth collected. The IgM was purified and prepared as an immune complex with mouse antihuman λ as described⁴ for immunization of mice. Selection of hybridomas was then based on reactivity with IgMId and not with normal IgM by ELISA and for the ability to bind to tumor cells in the presence of normal human serum.¹³

Binding ELISA to Assess Specificity

A preliminary assessment of specificity was carried out using λId or a pool of three λ Bence-Jones proteins coated directly onto a microtitre plate at 100 ng/mL. Monoclonal antibody was then added and bound mouse Ig detected with horseradish peroxidase (HRP)-labeled rabbit antimouse Ig (Nordic Labs, Maidenhead, England). Binding to IgM was investigated by the indirect procedure: rabbit antihuman μ chain (10 $\mu\text{g}/\text{mL}$) was coated onto the plate and IgM preparations, and either "rescue" hybridoma supernatants or macroglobulinemic sera were added, followed by MoAb as above.

Inhibition ELISA to Quantitate Relative Binding

This was established to measure the relative abilities of different λ -containing molecules to bind to the MoAb-Id(λ) and also to find the proportion of λId among normal λ chains. First λId was bound directly to the microtitre plate at 100 ng/mL, then a binding curve was constructed for anti-Id (λ) using HRP-rabbit antimouse Ig for detection. Having established the steepest part of the binding curve (40 ng/mL of antibody), this concentration of antibody was mixed with different dilutions of each inhibitor, and the resulting reduction in mouse antibody bound to the plate was measured. The concentration of inhibitor giving a 50% reduction in binding could then be compared for each molecular species, and the slope of the inhibition curves could indicate antigenic similarity. A major advantage of the

inhibition assay is that all inhibitors are compared in the fluid phase with no complications due to different coating efficiencies or to possible structural distortions occurring during binding to a solid phase.

Immunofluorescence

Testing of anti-idiotypes on the patient's dispersed tumor cells was by flow cytometry on the FACS III (Becton Dickinson, Mountain View, CA). For inhibition studies, normal human serum (50 μL) was added to culture supernatants (50 μL) or to purified antibody at $\sim 20 \mu\text{g}/\text{mL}$, before adding to the cells. Bound antibody was detected with fluorescent rabbit antimouse Ig.

Tissue Sections

Frozen tissue sections were used to assess reactivity of the anti-Id(λ) with normal tonsil and lymph nodes from nine patients with lymphoma. Cryostat sections (5 μm) were air-dried and stored at -70°C until required. Immediately before staining the sections were fixed in acetone for ten minutes and then stained by a two-layer immunoperoxidase technique,¹⁴ using HRP-rabbit antimouse Ig as the second layer and developing reaction product with diaminobenzidine tetrahydrochloride.

RESULTS

Production of λId by Tumor Cells

Neoplastic B cells from LP secreted in vitro free λ light chain (20 ng/h) and IgM (7.2 ng/h), values in the range of those found previously for patients with CLL.¹⁵ The levels of light chain (κ and λ) in a typical 24-hour urine sample from LP were 0.3 (κ) and 6.8 (λ) mg per 24 hours, which is below the detection level of most routine methods and requires sensitive IEF and immunofixation or other special techniques¹⁶ for identification. Identification of the monoclonal λ chain for LP by this technique has been reported.⁸ After purification of the urinary λId and passage through G-100 in 1 mol/L propionic acid, the disulfide-bonded dimer accounted for about 20% of total λ .

Monoclonal Anti-Id (λ)

In the first screen of selected hybridoma supernatants, seven clones were found to recognize λId and not pooled λ

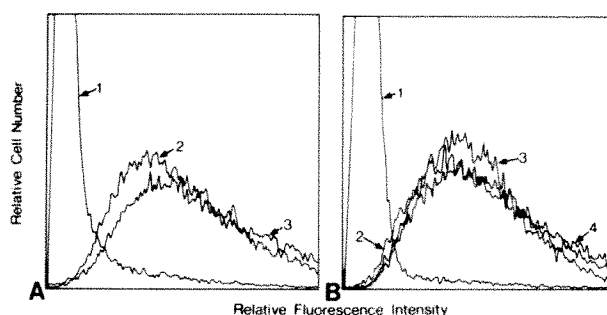


Fig 1. Binding of MoAbs to target lymphoma cells from patient LP. Tumor cells were exposed to mouse MoAbs, and bound mouse IgG was detected with fluorescent rabbit antimouse Ig. (A) 1: Anti-human κ chain (10 $\mu\text{g}/\text{mL}$); 2: anti-human λ chain constant region (10 $\mu\text{g}/\text{mL}$); 3: antibody against heavy plus light chain idiotypic (anti-Id [$\mu + \lambda$] (10 $\mu\text{g}/\text{mL}$)). (B) 1: Anti-human κ chain (10 $\mu\text{g}/\text{mL}$); 2: anti-human λ chain constant region (10 $\mu\text{g}/\text{mL}$); 3: antibody against λ chain idiotypic (anti-Id [λ] (24 $\mu\text{g}/\text{mL}$); 4: anti-Id (λ) plus normal human serum (50%).

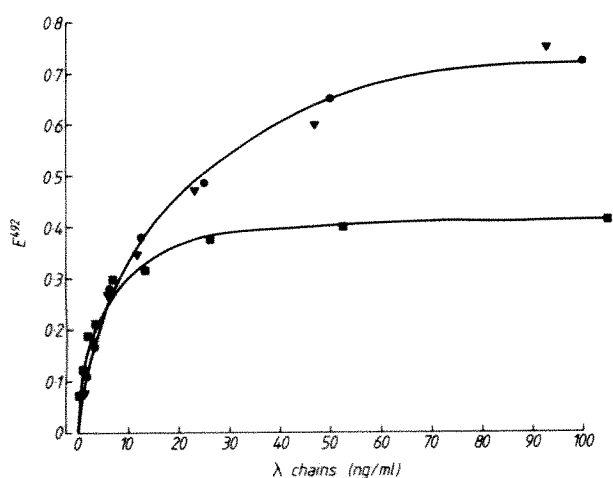


Fig 2. Comparison of binding of monomeric and dimeric λ chains to immobilized anti- λ constant region antibody. Rabbit antihuman λ chain constant region ($5 \mu\text{g/mL}$) was coated to the wells of a microtitre plate and exposed to various concentrations of idiotype λ chains from patient LP, previously separated into monomer and disulfide-bonded dimer. Detection of bound λ chain was by HRP-rabbit antihuman λ chain. ■—■, monomeric λ Id; ●—●, dimeric λ Id; ▼—▼, pool of three Bence-Jones proteins of λ type.

chains. Of these, three also recognized IgMId and not normal IgM, and the most vigorous of these was selected for expansion. Further tests of specificity showed the antibody to be unreactive with rescued IgM preparations (3 IgM λ) or 23 sera from macroglobulinemic patients (10 IgM κ and 13 IgM λ). The subclass of the antibody was IgG1.

Immunofluorescence showed good reactivity with tumor cells from LP, which was not affected by the presence of normal human serum (50%) (Fig 1). The antibody did not react with frozen sections of normal tonsil nor with nine lymph node specimens from patients with B cell lymphoma; antibodies to Ig constant region reacted normally with these sections.

Monoclonal Anti-Id ($\mu + \lambda$)

This antibody was selected by the usual criteria for anti-idiotypic considered for therapy, ie, it recognized IgMId

but not normal IgM by ELISA, and its binding to tumor cells from LP (Fig 1) was not inhibited by normal serum (50%). This antibody did not bind to λ Id by ELISA, and its binding to IgMId was unaffected by λ Id up to a concentration of $50 \mu\text{g/mL}$.

Nature of Determinant Recognized by Anti-Id(λ)

Monomeric and dimeric λ Id. It was of interest to determine the reactivity of the monoclonal anti-Id (λ) with monomeric or dimeric λ Id to see if the relevant determinant was more exposed on the monomer. Disulfide-bonded dimer and monomer were first assayed by ELISA using anti- λ constant region antibody on the plate and detecting bound λ with HRP-anti- λ . Results are shown in Fig 2 where it is clear that at saturation, monomer gives half the binding of dimer at equivalent concentrations. This is consistent with equal molar binding to the plate, with each bound dimer offering twice as many determinants as monomer to the detecting HRP-antibody. Apparently the monomer has not dimerized by noncovalent bonds under the conditions of the ELISA, presumably due to the routine presence of non-ionic detergent, Tween 20 (0.1%). A pool of three λ Bence-Jones proteins showed binding similar to that of λ Id dimer (Fig 2), suggesting that they are dimerized by disulfide bonds as has been found for most human λ chains.¹⁷

Comparison of the ability of the monomer and dimer to bind to the anti-Id (λ) was made by the inhibition ELISA (Fig 3). It was found that λ Id monomer was approximately $12\times$ more efficient than dimer on a weight basis ($6\times$ on a molar basis) in displacing the monoclonal anti-Id (λ) from binding, since the monomer caused 50% inhibition at 1.6 ng/mL , whereas 18.5 ng/mL of dimer was required (Table 1). There is also a deviation from parallelism for the dimer (Fig 3), suggesting that the antigenic determinant is not identical to that of the monomer.

Normal pooled λ chains. Reactivity of the anti-Id(λ) with normal λ chains was also quantitated by the inhibition ELISA (Fig 3). These λ chains were obtained from normal IgG λ by reduction and alkylation of the disulfide bonds followed by separation in 1 mol/L propionic acid and are therefore monomeric. It was found that $3.3 \times 10^4 \text{ ng/mL}$

Fig 3. Inhibition ELISA to compare the abilities of various λ -containing molecules to bind to monoclonal anti- λ chain idiotype. The assay depends on competing λ -containing molecules that bind in the fluid phase to the mouse anti-idiotypic antibody, thereby preventing its uptake by idiotype λ chain (λ Id) immobilized on the plate. The percentage reduction in bound mouse antibody was quantitated by HRP-rabbit antimouse Ig. ■—■, monomeric λ Id; ●—●, dimeric λ Id; ▼—▼, normal human monomeric λ chains; □—□, idiotype IgM λ .

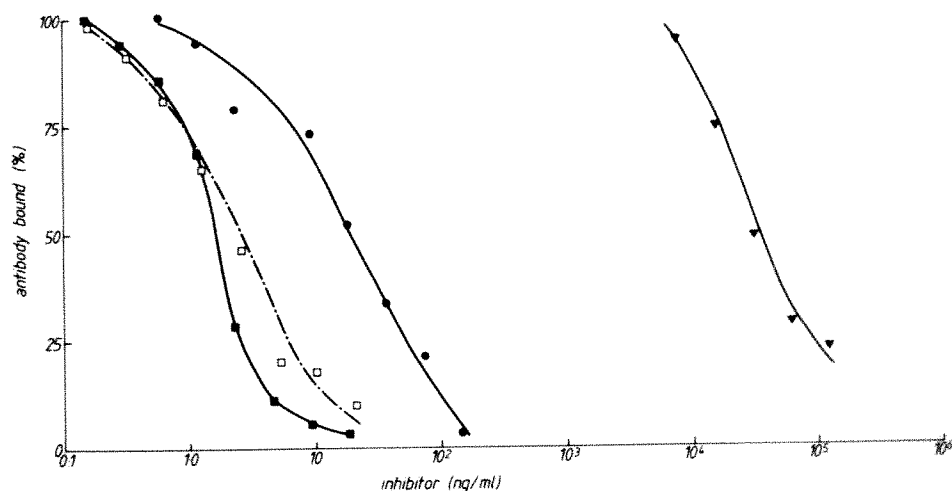


Table 1. Measurement of the Ability of Various λ -Containing Molecules to React with Monoclonal Anti- λ Idiotype (Anti-Id (λ)) by an Inhibition Assay*

Inhibitor	Molecular Form	Concentration for 50% Inhibition (ng/mL)
Idiotypic λ (λ Id)	Monomer	1.6
	Dimer	18.5
Normal λ	Monomer	3.3×10^4
Idiotypic IgM† (IgMId)	Pentamer	2.5

*For this assay, λ Id was coated to the wells of a microtitre plate and mouse anti-Id (λ) at 40 ng/mL was added, either alone or mixed with different dilutions of each inhibitor. The resulting reduction in mouse antibody bound to the plate was measured and the concentration of inhibitor giving a 50% reduction in binding found.

†Concentration is in terms of the λ chain content.

was required for 50% inhibition (Table 1), ie, only one molecule in about 20,000 carries the same idiotypic determinant as the λ Id.

Tumor-derived IgMId. The rescued IgMId was found to be predominantly pentameric from its elution curve on AcA 22; its ability to displace the anti-Id(λ) from its binding to λ Id antigen is shown in Fig 3. The amount of IgMId is given in terms of the content of λ chain for direct comparison, and it can be seen (Table 1) that 2.5 ng/mL is required for 50% inhibition, a value close to monomer and much more efficient than dimeric λ Id.

It was also found that normal serum (1 in 10) failed to inhibit binding, whereas LP's serum inhibited binding by 50% at 1 in 640 dilution.

Additive binding to IgMId by anti-Id (λ) and anti-Id ($\mu + \lambda$). This was investigated by ELISA using limited antigen on the plate (25 ng/mL of IgMId coated indirectly) and finding a saturating concentration of anti-Id ($\mu + \lambda$) (2.7 μ g/mL). The anti-Id (λ) was then added to this in increasing amounts to see if additional binding could occur; the results are shown in Fig 4. Clearly the binding of anti-Id (λ) is independent of the presence of anti-Id ($\mu + \lambda$), since the binding curve is very close to the calculated additive curve. The graph also illustrates that the avidity of the anti-Id(λ) is considerably less than that of the highly avid anti-Id ($\mu + \lambda$), since the latter reached saturation of bound IgMId at 2.7 μ g/mL, whereas the former was still not saturating at 100 μ g/mL. However, the amount required for binding to cells (Fig 1) was 24 μ g/mL, well within a useable range. There was no significant binding of an irrelevant mouse MoAb at these concentrations.

DISCUSSION

A monoclonal anti-idiotypic antibody has been raised against tumor-derived λ light chain from a patient with CLL. The antibody is specific for idiotypic determinants on the λ chain but also recognizes λ chain in combination with μ chain either as soluble idiotypic IgM or on the surface of tumor cells. These properties are similar to those of polyclonal anti-idiotypes previously raised against tumor-derived light chains from four patients with CLL⁹ and demonstrate that this dual recognition is due to a single antibody. The study also confirms the generality of the approach and, since clones

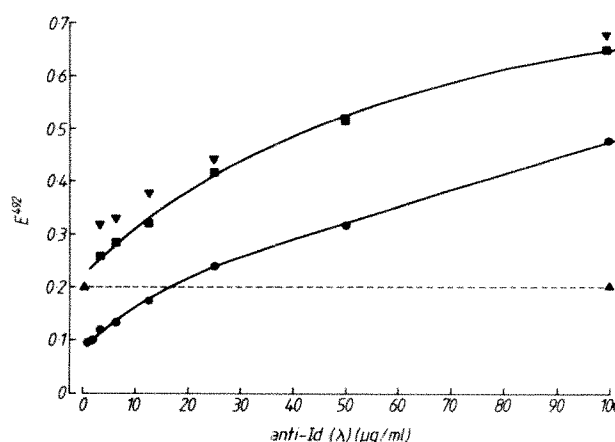


Fig 4. Additive binding of two monoclonal anti-idiotypes to idiotypic IgM. The ability of anti- λ idiotype (anti-Id (λ)) to bind to target IgMId in the presence of a saturating amount of anti-heavy-plus-light chain idiotype (anti-Id ($\mu + \lambda$)) was assessed. Limited antigen (25 ng/mL of IgMId) was coated to the plate indirectly and then exposed to increasing concentrations of anti-Id (λ) in the presence of anti-Id ($\mu + \lambda$) at 2.7 μ g/mL. Total bound mouse antibody was detected by HRP-rabbit antimouse Ig. ▲---▲, anti-Id ($\mu + \lambda$) (2.7 μ g/mL) alone; ●---●, anti-Id (λ) alone; ■---■, anti-Id (λ) in the presence of anti-Id ($\mu + \lambda$); ▼---▼, calculated values for additive binding.

showing dual specificity were not rare, suggests the possibility of using such antibodies clinically.

However, there are two possible problems. First, more aggressive immunization is required for light chain as compared to IgM.⁹ This could perhaps be solved by coupling light chain to a carrier such as keyhole limpet haemocyanin. The second problem is that the antibody studied had a considerably lower avidity for idiotypic IgM than did the antibody against heavy plus light chain (combined) idiotype, the latter having a very high avidity. Since antibody avidities are variable, this need not be a general property, but more antibodies need to be studied.

Using this MoAb it has been possible to investigate further the nature of the antigen recognized. In the more common situation where antibodies are raised against combined idiotype, they rarely bind to free light chain, more often recognizing determinants dependent on a conformation involving heavy and light chains.¹⁰ This has also been found for anti-idiotypes raised against IgM idiotype in this laboratory, including the one described in this report. Thus the idiotypic determinants present on the isolated light chains that can induce antibodies that recognize IgM, and therefore must be displayed on the IgM molecule, are either pre-empted by other immunogenic structures or are not detected in the assay systems used. One exception to the failure of anti-idiotypic raised against IgM to recognize free light chain is seen for rheumatoid factors (RFs). The existence of a major cross-reacting idiotype (Wa) among human monoclonal IgM anti-IgG autoantibodies from unrelated individuals with cryoglobulinemia was first described using polyclonal antisera.¹⁸ More recently a MoAb generated against this IgM idiotype was shown to react with isolated κ chains from those IgM-RFs expressing the idiotype.¹⁹ The relevant deter-

minant was localized to the second complementarity-determining region of the κ chain and antibody raised against the isolated peptide bound to the IgM-RFs.

The use of the inhibition ELISA has allowed quantitation of the degree of binding of the antibody to various λ -containing molecules. The first point is that since it only recognized 1 in 20,000 molecules of normal λ , it can be considered to be idiotype specific. Second, binding by monomeric λ Id, which in antigen excess could be twice as efficient as dimer on a weight basis if the determinant is equally exposed on the two molecular species, is 12 times more efficient, indicating that masking or distortion of the determinant occurs during dimerization. However, the λ component of IgMId is almost as efficient as monomeric λ , suggesting that the determinant is exposed while λ Id is combined with μ chain and that, unlike the Mcg light chain studied by x-ray diffraction,²⁰ the dimer does not assume a similar

conformation to the μ - λ combination. The generality of this is unknown but raises the possibility that one of the reasons for the failure of polyclonal anti-IgM idiotypes to recognize free light chain could be due to the use of dimeric light chains in the test system.

Thus the earlier findings, reported in all four cases of CLL investigated⁹ and in an animal lymphoma,²¹ that polyclonal antibodies raised against idiotype light chains will recognize idiotype IgM have been confirmed using MoAb and should open the possibility of using such reagents for monitoring and perhaps treating B cell lymphoma.

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Studies Investigating Platelet Aggregation and Release Initiated by Sera From Patients With Thrombotic Thrombocytopenic Purpura

By John G. Kelton, Jane C. Moore, and William G. Murphy

Many patients with thrombotic thrombocytopenic purpura (TTP) have a platelet aggregating factor in their serum that may be pathologically linked with the disease process. To help characterize the type of platelet aggregation and platelet release induced by the sera from seven TTP patients, we measured the ability of a variety of inhibitors of platelet function as well as the ability of monoclonal antibodies (MoAbs) against platelet glycoproteins to inhibit TTP sera-induced platelet aggregation and release. These results were compared with the ability of the same inhibitors to block platelet aggregation induced by ristocetin, collagen, ADP, thrombin, and IgG-immune complexes. Monoclonal antibody directed against platelet glycoprotein Ib totally inhibited ristocetin-induced aggregation and release but had no effect on aggregation and release induced by the TTP sera or by any of the other platelet agonists. However, the MoAb against glycoproteins IIb/IIIa inhibited aggregation and release caused by TTP sera as well as by collagen, thrombin, and ADP but had no effect on

aggregation and release induced by ristocetin. The aggregating activity could be abolished by heparin but not by the serine protease inhibitor PMSF (1 mmol/L). And although monomeric human IgG and purified Fc fragments of IgG inhibited IgG-immune complex-induced aggregation and release, they had no effect on TTP sera-induced aggregation and release nor on aggregation and release induced by any of the other agonists. Consistent with these in vitro studies showing no effect of IgG were the in vivo observations that intravenous (IV) IgG was without effect when administered to three patients with TTP. This study indicates that although a von Willebrand factor (vWF)-rich preparation of cryoprecipitate enhances the in vitro platelet aggregation and release caused by sera from the seven TTP patients we studied, the pathway of aggregation and release is not via platelet glycoprotein Ib. Also the aggregating factor of TTP sera is not neutralized in vitro or in vivo by IgG.

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THROMBOTIC thrombocytopenic purpura (TTP) is a life-threatening disease characterized by consumptive thrombocytopenia, schistocytic hemolytic anemia, fever, and acute arterial thrombotic complications especially within the cerebral circulation.¹ The thrombotic complications are due to the occlusion of small arteries by platelet thromboemboli. The pathogenesis of TTP remains uncertain, but the consumptive thrombocytopenia plus platelet thromboemboli suggest that in vivo platelet aggregation could participate in its pathogenesis. It is possible that the platelet aggregation could be mediated by a platelet aggregating factor with participation of von Willebrand factor (vWF).² A platelet aggregating factor in plasma or sera from patients with TTP has been described by two groups of investigators;^{2,3} however, the in vitro characteristics are not identical.²⁻⁴ There is also evidence that the vWF actively or passively participates in the pathogenesis of TTP. For example, Moake and colleagues described alterations in the vWF molecule in patients with TTP,^{5,6} observations confirmed by ourselves.² But again there is not total agreement about these observations.⁴

To study TTP sera-induced platelet aggregation in greater detail, we investigated whether monoclonal antibodies

(MoAbs) against platelet glycoprotein Ib and against glycoprotein IIb/IIIa could inhibit platelet aggregation and release initiated by TTP serum. Controls included platelet agonists that agglutinate platelets via platelet glycoprotein Ib (ristocetin) and other agonists (collagen and thrombin) that require glycoproteins IIb/IIIa for aggregation and release. The results of the study indicate that in vitro TTP sera-induced platelet aggregation and release occurs via glycoproteins IIb/IIIa and not Ib, and the platelet reaction is not affected by IgG.

MATERIALS AND METHODS

Patients and Controls

Sera from seven unselected patients with TTP were studied during the acute episodes before treatment. All patients had thrombocytopenia and schistocytic hemolytic anemia. In five of the patients, the pattern of vWF was analyzed by crossed immunoelectrophoresis before treatment was initiated. In all of these patients the large multimers of vWF were reduced or absent. Five of the patients had neurologic signs. One patient died, and postmortem examination confirmed the diagnosis as TTP.

Whole blood was collected into glass Vacutainer tubes (Becton Dickinson, Mississauga, Canada) containing no anticoagulant. The blood was allowed to clot spontaneously for a minimum of two hours and a maximum of 12 hours. The blood was centrifuged (2100 g for 20 minutes); the serum was transferred to plastic tubes and stored in aliquots at -70 °C. Before testing, the serum was thawed at 37 °C and then incubated for an additional 30 minutes at 37 °C to inactivate thrombin, and the pH was adjusted to 7.4.

Control sera were collected from healthy laboratory volunteers receiving no medications. This study was performed in accordance with the guidelines developed by the University-approved Ethics Review Committee.

Aggregating Agents

Platelet aggregation and release was induced using bovine achilles tendon collagen (final concentration, 55.1 µg/mL; Sigma Chemical Co, St. Louis), thrombin (final concentration, 2 U/mL; Fibrinex,

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Ortho Diagnostics, Westwood, MA), adenosine diphosphate (ADP; final concentration, 10 $\mu\text{mol/L}$; Sigma), ristocetin (final concentration, 1.5 mg/mL; H. Lundbeck & Co, Copenhagen), and IgG immune complexes.

IgG immune complexes were prepared from human IgG. The human IgG was isolated from normal AB sera twice precipitated with 50% ammonium sulphate followed by a diethylaminoethyl (DEAE) Sephacel column (Pharmacia, Dorval, Quebec). The IgG was stored at -70°C at a final concentration of 10 mg/mL. IgG immune complexes were made by heating 0.5-mL aliquots (63 $^\circ\text{C}$ for 20 minutes). Before using, the largest complexes were removed by centrifugation at 10,000 g for five minutes. Purified Fc fragments were prepared from monomeric IgG using papain (100:1, wt/wt). Undigested IgG was separated from Fc by gel chromatography, and the completeness of separation was verified by analytic polyacrylamide gel electrophoresis (5% SDS-PAGE). The concentration of Fc was raised to 20 mg/mL by ultrafiltration and the sample frozen at -70°C before use.

Inhibitors of Platelet Aggregation/Release

The effects of aspirin (3.3 mmol/L), EDTA (1.6 mmol/L) and IgG (5 mg/mL) were tested for their ability to inhibit platelet aggregation and release.

Murine MoAbs 6D1 against human glycoprotein Ib, (IgG₁, final concentration 10.5 $\mu\text{g/mL}$) and 7E3 against IIb/IIIa (IgG₁, final concentration 15 $\mu\text{g/mL}$) were also used.⁷ These MoAbs were a gift from Dr B. Collier of the State University of New York at Stony Brook. A control MoAb lacking platelet glycoprotein activity was also tested at a final concentration of 15 $\mu\text{g/mL}$.

The effect of two protease inhibitors, heparin (0.5 U/mL), and phenylmethylsulfonylfluoride (PMSF) (1 mmol/L; Sigma) were tested for their ability to inhibit TTP-induced platelet release.

Performance of Platelet Aggregation and Release Studies

Preparation of test platelets. Twenty milliliters of whole blood was collected from different healthy donors into acid citrate dextrose, pH 4.5, (6:1, vol/vol). The platelet-rich plasma (PRP) was obtained by centrifugation (160 g for 20 minutes). The PRP was incubated with ^{14}C serotonin (Amersham, Oakville, Ontario) for 30 minutes at 37°C at a concentration of 0.5 μCi ^{14}C serotonin per mL PRP. Approximately 50% of the ^{14}C serotonin was taken up by the washed platelets giving a final specific activity of 1.84×10^{-4} μCi of ^{14}C serotonin per 10^6 platelets. The platelets were pelleted by centrifugation (1800 g for nine minutes) and resuspended in 10 mL of calcium and albumin-free Tyrode's solution, pH 6.2, containing 50 μL of apyrase. The apyrase (50 $\mu\text{g/mL}$) was prepared using standard techniques and inhibited aggregation induced by 10 $\mu\text{mol/L}$ ADP. The platelets were washed once by centrifugation (1200 g for seven minutes) and resuspended to a final count of 600,000/ μL in albumin-free Tyrode's solution, pH 7.4. Because the reactivity of the platelet preparations occasionally differed from donor to donor, platelets were collected from three ABO compatible donors, pooled and processed as described above.

In a second series of experiments the platelets were resuspended with human fibrinogen (Kabi, Sweden) at a final concentration of 2 mg/mL. In these studies the assay was performed at 37°C and the effects of inhibitors of aggregation and release investigated as described subsequently. In these experiments the cryoprecipitate preparation was not added.

Preparation of solution containing large multimers of vWF. Two units of cryoprecipitate prepared from healthy donors were pooled and diluted with 10 mL of 0.9% sodium chloride. The cryoprecipitate was defibrinated using 0.15 units anecrod (Arvin,

Connaught Labs Ltd, Willowdale, Ontario) per millimeter of dilute cryoprecipitate. The mixture was incubated for ten minutes at 37°C , centrifuged (2100 g for 20 minutes), and heat-inactivated for 30 minutes at 56°C . The solution was stored at -70°C before using. Immediately before using the solution was thawed at 37°C and then subjected to centrifugation (12,000 g $\times$ 10 minutes) to remove large aggregates. We have previously demonstrated that this preparation contains large multimers of vWF.²

Platelet release studies. Twenty microliters of test serum (pH 7.4) was mixed with 60 μL of ^{14}C -serotonin-labeled platelets. The platelet mixture was incubated at room temperature in microtitre wells containing a magnetic stir bar. Following a ten-minute incubation, 3 μL of the preparation containing the large multimers of vWF was added (final concentration of 0.05 units). The suspension was stirred for an additional ten minutes. Both incubations were done on a magnetic stir plate set at a slow speed. At the end of the final incubation period, 100 μL of 0.5% EDTA in saline was added to terminate the release reaction. The platelets were then observed for evidence of visible aggregates and scored as positive (aggregates) or negative (no aggregates). The microtitre plates were centrifuged for five minutes at 1500 g and 50 μL of the supernatant fluid was removed. This aliquot was added to 10 mL of scintillation fluid and the samples were counted in a liquid scintillation counter. The percent release was calculated as follows:

Percent Release =

$$\frac{\text{Release (test sample)} - \text{Background}}{\text{Total Platelet Radioactivity} - \text{Background}} \times 100$$

The background was defined as the supernatant fluid radioactivity from platelets handled identically to the test platelets except that buffer was substituted for serum. Total radioactivity was the radioactivity of the test platelet sample. A test result was defined as positive if there was greater than 20% release. All samples were tested in duplicate.

Inhibition of platelet release and aggregation. The experiments were performed as follows: 10 μL of the potential inhibitor (EDTA, aspirin, heparin, PMSF, IgG, Fc fragments, anti-GP Ib, anti-GP IIb/IIIa, or buffer) was added to 60 μL of the platelet suspension followed by a ten-minute incubation at 22°C . The TTP serum (20 μL) or agglutinating agent (20 μL of ADP, collagen, ristocetin, thrombin, or IgG immune complexes) was then added to the mixture, and it was stirred for ten minutes at 22°C . The fibrinogen-depleted cryoprecipitate was added and mixed for an additional ten minutes at 22°C . One hundred μL of 0.5% EDTA in saline was added to terminate the reaction. The microtitre plates were centrifuged, and the radioactivity of the supernatant was measured.

To investigate whether IgG prepared from normal pooled sera or control sera could inhibit TTP sera-induced platelet release, the following experiments were performed: The amount of platelet release initiated by four different TTP sera or one normal control serum was measured using the technique described previously. Control IgG at a final concentration of 1.2 mg/mL or control serum was incubated with each TTP serum for 20 minutes at 22°C . The ^{14}C -serotonin labeled platelets (60 μL) were added, and, after an additional 20-minute incubation at 22°C , the amount of release was measured. In these experiments the four TTP sera that were used were selected because their strong reactivity did not require the addition of the preparation rich in vWF to induce the release reaction.

The same experiment was repeated using the same TTP and control sera except that the IgG or control serum was preincubated with the platelets (22°C for 20 minutes) before addition of the TTP sera.

RESULTS

Case Report 1

A 45-year-old woman was admitted with diagnosis of TTP and was treated with plasma exchange, aspirin, and dipyridamole. After an initial partial response the illness relapsed. The patient was treated with 23 different plasma exchanges but did not respond. She also did not respond to 4 mg of vincristine. Intravenous (IV) IgG (Gamimune, Cutter Biologicals, Berkeley, CA) was administered over two days at a dose of 2 g/kg. There was no effect on either the platelet count or on the serum LDH level. The patient died two weeks later of intracerebral thrombosis complicated by hemorrhage. Postmortem examination confirmed the diagnosis of TTP.

Case Report 2

A 54-year-old woman was admitted with TTP. She had had an episode of TTP five years previously that had responded to plasma infusion and plasmapheresis and had been well for the subsequent five years. On this admission she was treated with both plasma infusion and plasmapheresis, and her platelet count was maintained above 150,000/ μ L by weekly 3-L plasma exchanges. Because of difficulties in maintaining vascular access, she was treated with IV IgG (IVEEGAM; Immuno, Vienna, Austria) at a dose of 2 g/kg. She did not respond, and her platelet count fell to 50,000/ μ L over the next week. She was again treated by a plasma exchange, and her platelet count promptly rose. She is currently maintained by weekly plasma exchanges.

Case Report 3

A 5-year-old girl with congenital chronic relapsing TTP has been treated over the past five years with intermittent (usually three per year) plasma infusions when her platelet count falls below 50,000 per μ L. This treatment results in a

prompt rise in platelet count and hemoglobin level that persists for weeks to months. She responds to plasma, stored plasma, and cryoprecipitate infusions. On one occasion she was treated with IV IgG at a dose of 3 g/kg (Sandoglobulin; Sandoz Laboratories, Dorval, Quebec) but did not respond to the treatment. Subsequent treatment with plasma infusions promptly raised the platelet count and hemoglobin level. Serum from this child was not used in the *in vitro* studies described in this report.

Results of the Platelet Aggregation and Release Studies

The results of the platelet aggregation and release studies are shown in Table 1. Collagen-induced aggregation and release were not affected by aspirin, heparin, or the MoAb against platelet glycoprotein Ib. It was totally inhibited by the MoAb against glycoproteins IIb/IIIa. ADP reacted similarly. Thrombin-induced aggregation and release were inhibited by heparin and by the MoAb against glycoprotein IIb/IIIa.

Ristocetin-induced aggregation and release were only inhibited by the MoAb against glycoprotein Ib. Purified Fc fragments were without effect. IgG-immune complex-induced aggregation and release were totally inhibited by monomeric IgG and purified Fc. TTP sera-induced aggregation and release were unaffected by aspirin and totally inhibited by heparin (high concentrations) and EDTA. It was not affected by the MoAb against glycoprotein Ib but was inhibited by the MoAb against IIb/IIIa. It was not affected by either monomeric IgG or purified Fc. An additional five IgG fractions were also tested for inhibitory capacity, including IgG obtained from a patient with two previous episodes of TTP and who was in remission. None of these IgG fractions had any effect on TTP sera-induced platelet aggregation and release.

The study investigating the inhibitory effect of control IgG or serum preincubated with the TTP sera showed that

Table 1. Results of Platelet Aggregation and Release Studies

Agonist	Buffer	Biological Inhibitors of Platelet Aggregation				Chemical Inhibitors of Platelet Aggregation		
		Monoclonal Antibodies		IgG	Fc	Heparin	ASA	EDTA
		Ib	IIb/IIIa					
TTP								
#1	+(73)	+(100)	-(0)	+(68)	+(57)	-(8)	+(98)	-(4)
#2	+(55)	+(75)	-(18)	+(100)	+(100)	-(1)	+(100)	-(6)
#3	+(100)	+(100)	-(18)	+(100)	+(100)	-(5)	+(100)	-(17)
#4	+(57)	+(82)	-(2)	+(100)	+(92)	-(0)	+(100)	-(8)
#5	+(95)	+(88)	-(4)	+(90)	ND	-(7)	+(87)	-(0)
#6	+(90)	+(86)	-(0)	+(93)	ND	-(0)	+(83)	-(0)
#7	+(74)	+(70)	-(0)	+(62)	ND	-(0)	+(76)	-(10)
ADP	+(79)	+(89)	-(17)	+(100)	+(100)	+(80)	-(16)	-(20)
Ristocetin	+(100)	-(15)	+(100)	+(100)	+(100)	+(100)	+(100)	+(99)
Collagen	+(69)	+(100)	-(22)	+(70)	+(65)	+(65)	+(29)	+(29)
Thrombin	+(66)	+(77)	-(16)	+(83)	+(87)	-(1)	+(93)	-(4)
IgG Aggregates	+(90)	ND	ND	-(3)	-(11)	+(79)	+(100)	+(85)

The results of various inhibitors (shown along the top) on visible platelet aggregation (indicated by a + sign) and the percent ^{14} C serotonin release from platelets (noted within the brackets). The percent release was the mean of three or more experiments. The concentration of the aggregating agents and antagonists is given in the "Methods" section.

ND indicates not done.

neither control IgG nor serum affected the platelet release induced by the TTP sera. Similarly, preincubation of the test platelets with control IgG or serum did not block the effect of the TTP sera. The amount of platelet release initiated by the four different TTP sera with or without control IgG or serum ranged from 70% to 98%.

In the experiments in which fibrinogen was used at a physiologic concentration (2 mg/mL), the preparation rich in vWF was not used. Only three of the seven TTP serum samples caused release, and the amount of release was lower and ranged from 26% to 45% of maximum. However, the pattern of inhibition of release was identical to that noted in Table 1. In particular, the MoAb against platelet glycoprotein Ib was without effect, but the reaction was inhibited by the anti-IIb/IIIa antibody.

The TTP-induced platelet release could be entirely inhibited by heparin (0.5 U/mL). However, the aggregation was not caused by thrombin as shown by the lack of effect of PMSF. PMSF at a final concentration of 1 mmol/L had no effect upon the release reaction of seven different TTP sera. The release induced by these sera ranged from 64% to 100%.

DISCUSSION

The pathogenesis and optimal treatment of thrombotic thrombocytopenic purpura (TTP) remain uncertain. A number of hypotheses concerning the pathogenesis of TTP have been proposed: most are based on clinical observations, or on the results of *in vitro* studies, or on the response to certain therapeutic interventions. For example, untreated TTP has a mortality that may be as high as 90%.⁸ Treatment with antiplatelet agents reduces the risk of death, suggesting that *in vivo* platelet aggregation contributes to the morbidity and mortality of TTP.⁹ Supporting this hypothesis are postmortem reports of patients with TTP who have disseminated platelet thromboemboli in their small vessels.⁸ Also consistent are reports of platelet aggregating factors in the plasma of some patients with TTP.^{2,3} It is possible that by characterizing and identifying the aggregating factor, a more effective therapeutic strategy for dealing with this disorder might be developed.

In vitro and *in vivo* studies also support an active or passive role for vWF in the pathogenesis of the disorder. Moake and associates demonstrated abnormal patterns of vWF in patients with chronic relapsing TTP, an observation confirmed by ourselves.² Consistent with the hypothesis that vWF participates in the disease process are two other observations. First, we have shown that the *in vitro* reactivity of a platelet aggregating factor in the serum of some patients with TTP can be enhanced by the addition of a preparation rich in large multimers of vWF.² And second, pathologic studies have demonstrated the presence of vWF in occluded arteries from patients with TTP.¹⁰ Because vWF-induced platelet agglutination involves the platelet glycoprotein Ib, one might hypothesize that blocking this interaction might inhibit TTP sera-induced platelet aggregation and release. To investigate this issue more directly, we studied the effect of a variety of inhibitors of platelet aggregation on the aggregation and release induced by TTP sera. The results of

these aggregation and release studies were compared with results obtained using other platelet agonists. We found that the *in vitro* platelet aggregation and release initiated by TTP sera is mediated via platelet glycoproteins IIb/IIIa; and, as another group of investigators recently reported, glycoprotein Ib is not involved.⁴ Hence our results argue strongly against the reaction being identical to ristocetin-induced agglutination. The MoAb against glycoprotein Ib totally inhibited ristocetin-induced agglutination and release but had no effect on TTP, collagen, ADP, or thrombin-induced aggregation and release. In contrast, the MoAb against glycoprotein IIb/IIIa had no effect upon ristocetin-induced agglutination and release but inhibited the TTP sera-induced aggregation and release as well as the aggregation and release caused by the other agonists. Certain characteristics of TTP sera-induced platelet aggregation are similar to thrombin-induced platelet aggregation. In particular, it can be inhibited by heparin (Table 1). The observation raises the question of whether the TTP aggregating factor could be an artifact, such as thrombin, generated during blood collection. Two observations argue strongly against this possibility. First, as we previously demonstrated,² the thrombin-specific inhibitor DAPA was without effect. Second, PMSF, a serine protease inhibitor, also was without effect.

The observation that TTP sera-induced platelet release occurs through glycoprotein IIb/IIIa and not Ib should not be interpreted as indicating that vWF is not involved in the disease pathogenesis. It has been shown that vWF can also interact with glycoprotein IIb/IIIa, and modifications of the molecule dramatically increase its reactivity.^{11,12} Yet the mechanism of aggregation or characteristics of the inhibitory effect of normal plasma that result in remission in TTP remain uncertain. A role for IgG in either initiating the illness or controlling it has been proposed by one group.⁴ Elevated levels of platelet-associated IgG have been described in patients with TTP by several groups of workers, including ourselves; others have described circulating immune complexes in these patients. Together these observations might indicate that IgG immune complexes mediate the platelet aggregation. Indeed, IgG immune complexes are potent inducers of platelet aggregation, and aggregation induced by these complexes can be inhibited by IgG or the Fc portion of the molecule. However, our current *in vitro* and *in vivo* studies suggest that it is unlikely that IgG plays a causal or protective role in TTP. *In vitro* platelet aggregation initiated by IgG aggregates did not react similarly to the TTP sera-induced platelet aggregation in that the former could be blocked by either the addition of monomeric IgG or Fc. In contrast, both intact IgG and purified Fc were without effect upon TTP-induced platelet aggregation and release. These results are different from those obtained by another group of investigators studying TTP sera-induced platelet aggregation. They reported that IgG either from a control population or from a patient following recovery from TTP could neutralize the platelet aggregating factor.¹³ The reason for the difference in results between groups is uncertain and could be due to differences in experimental techniques. However, our *in vitro* data, failing to document a role for IgG in neutralizing TTP sera-induced platelet aggregation, is

further supported by the observation that high doses of IV IgG did not produce a remission in three patients with TTP. Although other investigators have reported an apparent response of TTP patients to IV IgG,¹⁴ such observations should be interpreted with caution. It is possible that the remission of the disease was spontaneous and only coincidentally linked to the IV IgG. It should be noted that two of the patients described in this report who failed therapy with IV IgG subsequently responded to more conventional therapy.

In summary, our studies demonstrate that TTP sera-

induced platelet aggregation and release is mediated via platelet glycoproteins IIb/IIIa. Although a preparation rich in vWF enhances the reaction in vitro, the pathway of release is not through glycoprotein Ib.

ACKNOWLEDGMENT

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Prognosis of Chronic Lymphocytic Leukemia: A Multivariate Regression Analysis of 325 Untreated Patients

By Jung S. Lee, Dennis O. Dixon, Hagop M. Kantarjian, Michael J. Keating, and Moshe Talpaz

Three hundred twenty-five previously untreated patients with chronic lymphocytic leukemia were analyzed to identify significant prognostic factors for survival. Univariate analysis identified the following characteristics associated with survival: (1) clinical characteristics: age, race, sex, performance status, lymphadenopathy, and hepatosplenomegaly; (2) hematologic parameters: WBC count, absolute lymphocyte and granulocyte counts, hemoglobin level, and platelet count; and (3) biochemical parameters: serum albumin, calcium, uric acid, lactate dehydrogenase, alkaline phosphatase, BUN, and creatinine. Multivariate regression analysis in a randomly selected training subset of 217 patients demonstrated that the combination of uric acid, alkaline phosphatase, lactate dehydrogenase, external lymphadenopathy, and age had the strongest predictive

relation to survival time. The resulting model was validated in the remaining independent subset of 108 patients and led to classification of patients into low, intermediate, and high-risk groups with five-year survival rates of 75%, 59%, and 14%, respectively, and with distinctively different annual mortality rates ($P < .01$). Both the regression model and Rai staging were highly effective in identifying risk groups among the entire patient population ($P < 0.001$). Overall the regression model was superior to Rai staging in defining prognostic risk groups. In addition, it was able to separate patients into significantly different risk categories within each Rai stage, thus improving on the prognostic prediction of individual patients with chronic lymphocytic leukemia.

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THE HIGHLY VARIABLE clinical course of chronic lymphocytic leukemia (CLL) poses difficulties in the evaluation of prognosis and the efficacy of therapy in individual patients as well as in clinical trials. Since the concept of clinical staging of CLL was introduced by Dameshek,¹ Rai et al² proposed a clinical staging system based on routine clinical and hematologic features that has subsequently been confirmed.^{3,4} Other studies have reported the importance of the absolute lymphocyte count, splenomegaly, hemoglobin levels, and the extent of lymphoid organ involvement in an attempt to improve this classification.⁵⁻¹¹ A new staging system, recommended by the International Workshop of CLL (IWCLL),¹² limits the clinical stages of CLL to three. All staging systems identify a high-risk group of patients defined by the presence of anemia or thrombocytopenia.⁵⁻¹⁴ In the remaining patients, who represent 70% to 80% of all cases, there is considerable variability in the course of the disease that is not well explained by the staging system. More recent studies have attempted to demonstrate the prognostic significance of immunologic parameters, bone marrow histopathologic pattern, and cytogenetics,¹⁵⁻²⁵ but the relative importance of each of these factors has not been determined.

The development of a prognostic model provides an objective estimate of an individual patient's prognosis, allowing group imbalance adjustments in comparing treatments and providing a basis for risk-directed therapy. Given the importance of adapting therapy to the severity of a disease that has a variable clinical course, we sought to identify significant prognostic factors and to develop a regression model aimed at stratifying patients according to prognosis for survival.

MATERIALS AND METHODS

Diagnostic Criteria

The diagnosis of CLL was based on the history, physical examination, peripheral blood smears, and bone marrow examination, with more than 15×10^3 lymphocytes/ μ L in the peripheral blood and at least 40% bone marrow lymphocyte infiltration.²⁶ Patients with poorly differentiated lymphocytic lymphoma, leukemic forms of well-differentiated diffuse lymphocytic lymphoma without initial bone marrow or peripheral blood involvement, prolymphocytic leu-

kemia, and lymphosarcoma cell leukemia were excluded from the study.

Patient Population

A total of 525 patients was registered at M. D. Anderson Hospital with the diagnosis of CLL between 1970 and 1983. Forty-seven patients had an incomplete evaluation mainly because of referral for other types of malignancy. Fourteen additional patients did not meet the diagnostic criteria on their initial evaluation, and follow-up information failed to confirm the diagnosis. One hundred thirty-nine patients had received treatment prior to admission. Thus 325 previously untreated patients were included in the analysis.

Among the 325 patients, 28% have never received any treatment after referral, 45% received single agent or combinations of conventional oral chemotherapy (chlorambucil, prednisone, and cyclophosphamide), and 27% received combination chemotherapies (adriamycin, cytosine arabinoside, cyclophosphamide, vincristine, and prednisone).

Prognostic Factor Analysis

Forty-one clinical and laboratory characteristics documented at initial evaluation were examined in the prognostic factor analysis. These were age, sex, race, year of diagnosis, interval between diagnosis and referral, performance status, symptoms (fever, weight loss), previous episodes of infection, hemolytic anemia or autoimmune thrombocytopenia, spleen and liver size (in cm below the costal margin), extent and site of lymphadenopathy (external, mediastinal, and retroperitoneal), hemoglobin (Hb) concentration,

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peripheral WBC and platelet counts, differential counts of WBC in peripheral blood, 12 channel-automated serum biochemical parameters, serum immunoglobulin levels, and bone marrow parameters including cellularity and differential count. In addition, the presenting Rai classification stage was considered.

Statistical Methods

Survival curves were plotted according to the method of Kaplan and Meier.²⁷ Comparison of curves was based on log-rank tests.²⁸ Prognostic-factor analyses are sometimes criticized because of failure to identify an independent population in which the regression model can be validated.²⁹ For this purpose we randomly divided the 325 cases into training and test subsets containing 217 and 108 cases, respectively, so that we could identify important prognostic factors in one set of data and validate them in a second.²⁹

Using patient characteristics identified in univariate analyses, a proportional hazards regression analysis³⁰ was carried out in the training set cases. We assumed that the hazard rate (the instantaneous risk of death among patients having survived until time t) has the form

$$\log \frac{\lambda_i(t)}{\lambda_0(t)} = \beta_1 z_{1i} + \beta_2 z_{2i} + \dots + \beta_p z_{pi},$$

in which $\lambda_i(t)$ is the hazard rate for the i th patient whose coded patient characteristics are $z_{1i}, z_{2i}, \dots, z_{pi}$. $\lambda_0(t)$ is the hazard rate for a patient for whom all characteristics are coded 0.

Measured characteristics were coded by initially dividing the cases into a maximum of five subgroups of about equal size and then pooling subgroups with similar survival curves.

To identify the characteristics to be included, we used the forward stepwise selection approximation suggested by Peduzzi et al.³¹ Characteristics entered the model according to their level of significance, after adjusting for the influence of the ones already selected, until the p -value for adding another characteristic exceeded 0.05. The data were then re-analyzed, with only the characteristics identified, using the more accurate maximum partial likelihood ratio statistics in the forward stepwise selection and stopping when the p value for adding an additional variable was above 0.01.

To assess the quality of the model, we: (1) divided the training set cases into three risk groups according to the fitted hazard rates and compared the corresponding survival curves; (2) repeated the procedure for test set cases; and (3) re-estimated the regression coefficients for the characteristics in the model based on cases in the test set. A characteristic was excluded from the model, and the whole process was restarted if the new estimate failed to exceed its standard error.

Smoothed estimates of annual mortality rates were derived as suggested by Simes and Zelen.³² The BMDP library of programs³³ was used for all analyses. The BMDP special function RNDU was used for the random assignment of cases to the training and test subsets.

RESULTS

The overall median survival from the date of referral to our institution was 5.4 years; 25% of patients survived beyond 10 years, while 10% of patients died within 1 year. At the time of analysis, 192 of 325 patients had died, and median follow-up among the 133 surviving patients was 5.2 years. Among the 150 patients with known causes of death, this was due to complication of advanced CLL in 33%; infections, predominantly pneumonia, in 25%; unrelated diseases (cardiovascular, renal) in 23%; second malignancy in 7%; other leukemias in 6%; and bleeding in 5%. Patient

characteristics of the training and test sets were comparable, and survival curves were almost identical (Fig 1).

Univariate Analysis of Prognostic Factors

Clinical characteristics. The univariate analysis of the clinical characteristics is presented in Table 1. The median age was 62 years (range 25 to 88 years), and survival progressively declined with advancing age. Males, representing 61% of our patients, and blacks, representing 9%, had significantly shorter survival than other patients. The number of patients referred to our institution was fairly even throughout the period, and there was no difference in survival by year of diagnosis, despite differences in therapy and supportive care throughout these periods of time. Fifty-six percent of patients were referred within 1 month of diagnosis and had significantly shorter median survival than others. Early referrals (within 1 month of diagnosis) tended to have advanced disease (Rai 3 or 4) more frequently than later referrals (32% v 13%). Poor performance status, symptomatic state, and weight loss were all associated with shorter survival. Patients who presented with external lymphadenopathy at any site greater than 1 cm, mediastinal lymphadenopathy, or hepatosplenomegaly had a significant survival disadvantage, although the degree of hepatosplenomegaly did not influence survival.

Hematologic characteristics. Hemoglobin concentration, platelet counts, WBC counts, absolute lymphocyte counts, and percentage of polymorphonuclear cells in the peripheral blood influenced survival. A distinct stepwise relationship was noted between the hemoglobin levels and survival but not between platelet count and survival. The absolute WBC counts and absolute lymphocyte counts in the peripheral blood were inversely associated with survival, whereas the percentage of polymorphonuclear cells was directly related to improved survival.

Serum biochemical parameters and immunoglobulins. All the biochemical parameters, except serum bilirubin levels, demonstrated a strong correlation with survival. Survival of 17 patients with monoclonal gammopathy was not significantly different from the survival of the remaining patients. The immunoglobulin levels, available for 210 patients without monoclonal gammopathy, failed to demonstrate any association with survival.

Bone marrow studies. Bone marrow differential counts were available in 185 patients and cellularity in 207. Bone

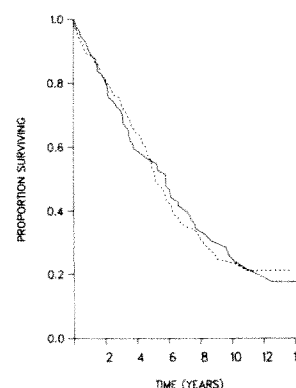


Fig 1. Overall survival of the studied patients with CLL. ---, Training set (N = 217); —, Test set (N = 108).

Table 1. CLL Univariate Analysis: Characteristics Influencing Survival

Characteristics	Category	No. of Patients	Median Survival Years	Significance Level
I. Clinical Features				
Age (in years)	≤50	42	9.0	<0.001
	51–60	99	6.2	
	61–70	120	5.2	
	>70	64	3.3	
Sex	Male	197	4.7	<0.001
	Female	128	7.4	
Race	Black	29	3.3	0.002
	Other	196	5.8	
Year of diagnosis	1970–74	135	5.3	0.83
	1975–79	132	5.6	
	1980 +	58	Not reached	
Duration since diagnosis	≤1 month	182	4.7	<0.001
	≥1 month	143	7.4	
Performance status (Zubrod scale)	0	287	5.9	<0.001
	≥1	37	1.8	
Weight loss	No	248	6.0	<0.001
	Yes	71	2.8	
External lymphadenopathy	No	80	10.2	<0.001
	Yes	245	5.0	
Mediastinal lymphadenopathy	No	289	5.8	0.008
	Yes	35	3.7	
Hepatomegaly	No	239	6.5	<0.001
	Yes	86	2.8	
Splenomegaly	No	198	7.1	<0.001
	Yes	126	3.8	
II. Hematologic Features				
Hemoglobin (g/dL)	<11	67	3.1	<0.001
	11–12.9	83	5.3	
	13–14.9	114	5.8	
	≥15	61	8.1	
Platelets (× 10/μL)	≤100,000	28	3.5	0.015
	>100,000	297	5.8	
WBC (× 10/μL)	≤25,000	109	9.0	<0.001
	25–50,000	94	6.3	
	>50,000	121	3.7	
Absolute lymphocyte counts (× 10/μL)	≤20,000	112	8.6	<0.001
	20–40,000	80	6.0	
	>40,000	132	3.9	
Percent of polys	≤5	78	3.1	<0.001
	6–20	144	5.6	
	>20	103	8.3	
III. Biochemical Features				
Albumin (g/100 mL)	≤4.5	170	4.4	0.010
	>4.5	148	5.9	
Calcium (mg/100 mL)	≤8.5	27	2.1	<0.001
	>8.5	290	5.8	
Uric Acid (mg/100 mL)	≤4	36	Not reached	<0.001
	4.1–6	124	6.6	
	6.1–8	123	4.8	
	>8	33	3.4	
BUN	≤20	233	6.2	<0.001
	>20	84	3.3	
Creatinine (mg/100 mL)	≤1.4	288	5.8	<0.001
	>1.4	29	2.1	
Bilirubin (mg/100 mL)	≤1.0	297	5.5	0.39
	>2.0	19	4.1	
Alkaline phosphatase (mU/mL)	≤80	180	6.3	<0.001
	>80	134	3.5	

(continued on following page)

Table 1. (Continued)

Characteristics	Category	No. of Patients	Median Survival Years	Significance Level
III. Biochemical Features (Continued)				
LDH (mU/mL)	≤225	217	5.9	<0.001
	226–350	65	4.8	
	>350	30	2.6	
SGOT (U/L)	≤20	129	5.8	0.038
	21–40	158	5.5	
	>40	23	5.2	
IV. Immunoglobulin				
Monoclonal gammopathy	No	210	5.8	0.97
	Yes	17	4.4	
IgG (mg/100 mL)	≤650	45	5.9	0.80
	651–1000	82	5.8	
	>1000	83	5.7	
IgA (mg/100 mL)	≤75	108	5.8	0.52
	>75	101	5.8	
IgM (mg/100 mL)	≤30	9	1.3	0.15
	>30	201	5.8	
V. Bone Marrow Features				
Cellularity (%)	≤50	97	8.3	<0.001
	50–90	82	5.1	
	>90	28	3.1	
Percent lymphoid	≤50	97	8.3	<0.001
	59–90	82	7.9	
	>90	28	2.2	
Percent myeloid	≤5	55	3.8	<0.001
	>5	130	8.3	
Percent erythroid	≤5	72	5.0	0.010
	5–10	45	6.5	
	>10	68	9.6	
VI. Rai Stage				
	0	58	Not reached	<0.001
	1	110	6.2	
	2	81	4.3	
	3	47	2.7	
	4	29	3.3	

marrow cellularity and percentage of lymphoid cells in the bone marrow differential were inversely associated with survival, while higher percentages of myeloid (>5%) and erythroid (>10%) cells had a significant positive influence on survival.

Rai stage. Survival by Rai stage is shown in Fig 2. Rai 0 patients have not reached a median survival. Median survivals of Rai 1 and 2 patients were 6 years and 4.2 years, respectively. There was no difference in median survival between Rai 3 and 4 patients.

Multivariate Regression Analysis

Forward stepwise selection-regression analysis, using the cases in the training set, resulted in selection of serum uric acid level, performance status, presence of external lymphadenopathy, serum creatinine level, and age, in the order listed, all with *P* values less than 0.01. This model, although confirmed in the test set of cases, depended on performance status, which is a subjective measurement, in a retrospective analysis of chart review.

A regression analysis, excluding performance status, selected the following: uric acid level, alkaline phosphatase

level, lactate dehydrogenase level, presence of external lymphadenopathy, and age, all with levels less than .01 (Table 2). Risk scores were calculated for each patient by using the regression equation (Table 2). Patient risk score distribution is shown in Fig 3. The training set was divided into three risk groups, using cut-points of 1.7 and 3.0. Survival curves for these low, intermediate, and high-risk groups are given in Fig 4. The regression model was validated

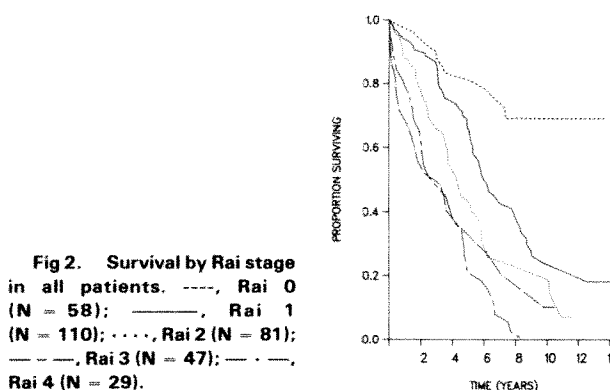


Table 2. Multivariate Analysis of Prognostic Factors in CLL

Characteristic	Code	Training Set	Testing Set	Total Population
I. Summary of Regression Model Analysis Relating Survival to Admission Features*				
Uric acid (mg/100 mL)	0 = ≤ 4 ; 1 = 4.1-6; 2 = 6.1-8; 3 = > 8	0.46 (0.12)	0.47 (0.16)	0.44 (0.10)
Alkaline phosphatase (mU/mL)	0 = ≤ 80 ; 1 = > 80	0.51 (0.19)	0.43 (0.27)	0.50 (0.15)
LDH (mU/mL)	0 = ≤ 225 ; 1 = 226-350; 2 = > 350	0.46 (0.13)	0.29 (0.19)	0.42 (0.11)
External lymphadenopathy	0 = no; 1 = yes	0.88 (0.25)	0.63 (0.33)	0.83 (0.20)
Age (in years)	0 = ≤ 50 ; 1 = 51-60; 2 = 61-70; 3 = > 70	0.28 (0.10)	0.53 (0.16)	0.35 (0.09)
II. Regression Model				
$\ln \frac{\lambda_i(t)}{\lambda_o(t)} = 0.44 \text{ (uric acid)} + 0.50 \text{ (alkaline phosphatase)} + 0.42 \text{ (LDH)} + 0.83 \text{ (external lymphadenopathy)} + 0.35 \text{ (Age)}$				

*Entries are regression coefficient and standard errors.

by demonstrating significant segregation of risk groups in the test set of patients ($P < 0.001$) (Fig 5).

Re-estimation of the regression coefficients for the five characteristics, using only the cases in the test set, shows comparable estimates to those of the training set, further confirming the importance of these variables in an independent population and validating the model (Table 2). The final estimates used in the regression equation are derived from the 307 patients with complete data from the combined training and test subsets (Table 2).

The prognostic significance of several patient characteristics available only on smaller subsets of patients (generally the more recent ones) was analyzed. Bone marrow biopsy findings were available on 185 patients. When various regression models were fit that included terms for uric acid level, external lymphadenopathy, alkaline phosphatase level, LDH level, age, and one or more of the bone marrow biopsy findings, only the percentage of myeloid cells made a significant but marginal contribution ($P = 0.035$).

Assignment of patients to risk groups using the detailed regression equation is inconvenient for routine clinical use. We examined several simplified alternative models in which all of the characteristics were limited to two categories. One of these, with uric acid coded 0 if ≤ 7.0 and 1 if > 7.0 , with LDH coded 0 if ≤ 325 and 1 if > 325 , and with age coded 0 if

≤ 60 and 1 if > 60 , produced a separation of survival curves into three groups that was nearly as good as for the detailed model. The new equation is: $\ln [\lambda_i(t)]/[\lambda_o(t)] = .68 \text{ (uric acid)} + 0.52 \text{ (alkaline phosphatase)} + 1.13 \text{ (LDH)} + 0.82 \text{ (external lymphadenopathy)} + 0.59 \text{ (age)}$.

Choosing cutpoints of 1.0 and 2.2 leads to risk groups having survival curves shown in Fig 6. The low-risk (score ≤ 1.0) group includes only patients with LDH ≤ 325 and no more than one unfavorable characteristic among uric acid, alkaline phosphatase, external lymphadenopathy, and age. The intermediate-risk (score 1.1 to 2.2) group includes patients with LDH > 325 and at most one unfavorable characteristic, or LDH ≤ 325 and two to three other unfavorable characteristics. The high-risk (score > 2.2) patients are those with LDH > 325 and two other unfavorable characteristics or those with any four or more unfavorable characteristics. Table 3 summarizes these criteria for easier clinical application.

Comparison of the Regression Model with Rai Staging

The distribution of patients by different risk groups and Rai stages is shown in Table 4. We evaluated the capacity of the regression model to identify groups of patients with different survivals within each of the Rai stages. As shown in Table 5, the regression model was useful in identifying patients at high risk even in the Rai 0, 1, and 2 categories. It

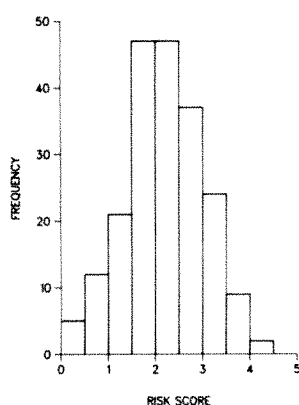
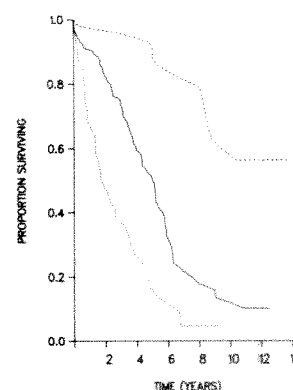


Fig 3. Distribution of risk scores in the training set.

Fig 4. Survival by risk score in the training set. ---, Risk score ≤ 1.7 (N = 52); —, Risk score 1.8 to 3.0 (N = 117); ····, Risk score > 3.0 (N = 35).



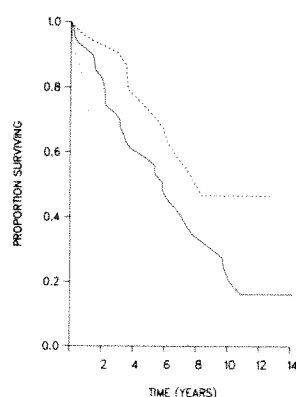


Fig 5. Survival by risk score in the test set. ----, Risk score ≤ 1.7 (N = 35); —, Risk score 1.8 to 3.0 (N = 50); ····, Risk score > 3.0 (N = 18).

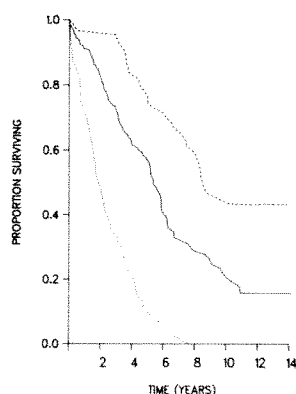


Fig 6. Survival by simplified risk score in all patients. ----, Low risk (N = 93); —, Intermediate risk (N = 166); ····, High risk (N = 48).

also identified patients with relatively better prognosis among Rai 1 to 2 or 3 to 4 stages, compared to their expected median survival by Rai stage.

Changes in Pattern of Referral and Influence of Therapy on Survival

The difficulties in evaluating the effect of therapy in patients referred over a period of 13 years and who have received multiple therapies sequentially for a chronic disease are obvious. Recently, patients with advanced Rai 3 and 4 disease have been treated with intensive combination chemotherapy such as prednisone, vincristine, cytosine arabinoside, cyclophosphamide, and adriamycin (POACH). The patterns of supportive care have also been relatively similar.

To suppose the possible effect of change of therapy, patients were divided into two equal groups, before 1976 and since 1976, which also corresponded to the time when more intensive regimens were being introduced for late-stage CLL. As shown, no change in the referral pattern by Rai staging was observed. Overall the median survival was similar in the two periods. More importantly, within early

Table 3. Criteria for a Simplified Risk Group Assignment

No. of Unfavorable Characteristics*	LDH ≤ 325	LDH > 325
0-1	Low	Intermediate
2-3	Intermediate	High
4	High	High

*Unfavorable characteristics: Uric acid > 7.0 mg/100 mL; alkaline phosphatase > 80 mU/mL; external lymphadenopathy present; and age > 60 years.

Table 4. Distribution of Patients by Risk Category and Rai Stages (307 Patients)

Rai Stage	No. of Patients (percent)		
	Risk Category		
	Low	Intermediate	High
0	41 (13)	16 (5)	—
1-2	35 (11)	125 (41)	18 (6)
3-4	11 (4)	34 (11)	27 (9)

and late Rai stages, by year of therapy (Table 6), no obvious change in median survival was observed either, indicating the stability of the population course despite the variability of therapeutic approaches over this period of time.

DISCUSSION

The identification of the prognostic factors that contribute to the heterogeneity of CLL disease is useful for individual prognostication and for therapeutic planning. This analysis of 325 previously untreated CLL patients from a single institution identified 29 characteristics strongly associated with survival duration. The study confirms the prognostic importance of several previously described features. The adverse prognosis of characteristics such as weight loss, performance status, hepatomegaly, splenomegaly, increased WBC and lymphocyte counts, elevated serum alkaline phosphatase, LDH, and uric acid levels probably reflect increased tumor burden. The poor prognosis associated with anemia and thrombocytopenia probably measures bone marrow function compromise secondary to the increased leukemic infiltrate.¹⁻¹⁴ Abnormal biochemical parameters such as the serum calcium, albumin, and BUN and creatinine levels had significant adverse influences on survival, possibly indicating a poor host biological status and organ dysfunction.

Monoclonal gammopathy, mostly involving IgM, was present in 7% of patients.³⁴⁻³⁶ The presence of monoclonal gammopathy and the level of serum immunoglobulins were not associated with survival differences.²⁴ Bone marrow disease features correlated with peripheral blood patterns. The extent of marrow involvement expressed by the cellularity and the percentage of lymphoid cells were inversely correlated with survival.^{37,38} While the importance of histo-

Table 5. Survival of Patients by Risk Category Within Rai Stages

Rai Stage	Risk Category	No. Patients	Median Survival (months)		Significance Level
			Rai	Risk	
0	Low	41	164+	164+	.0003
	Intermediate	16		40	
1	Low	23		100	.0001
	Intermediate	70	74	70	
	High	9		27	
2	Low	12		122	.017
	Intermediate	55	52	51	
	High	9		29	
3-4	Low	11		57	.02
	Intermediate	34	38	29	
	High	27		24	

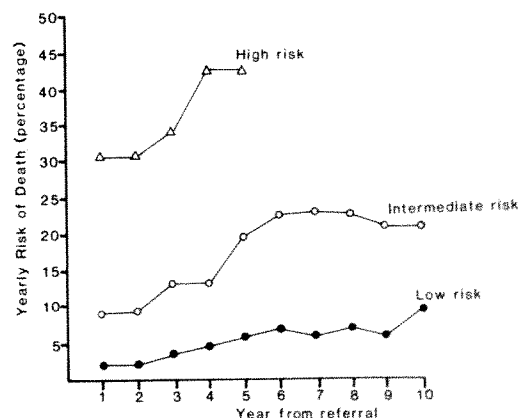
Table 6. Changes in Referral Patterns and Patient Outcome by Year of Therapy

Characteristic	Percentage of Patients		Significance Level
	1970-1975 (#172)	1976-1983 (#153)	
I. Rai Stage	No. (percent)	No. (percent)	
0-2	131 (76)	117 (77)	0.96
3-4	41 (24)	35 (23)	
II. Median Survival (Months)			
Overall	62	80	0.17
Rai 0-2	70	Not reached	0.10
Rai 3-4	29	37	0.65

pathologic patterns of bone marrow involvement, B cell markers, and cytogenetics have been appreciated recently,¹⁵⁻²⁵ these features were excluded from our analysis because they were available in only a small fraction of the patients.

Confirmation of the validity of a regression model in an independent population is essential for further clinical application.²⁹ In this study the regression model was derived from a training set of two thirds of patients and confirmed in the test set of the remaining one third. The derived regression model segregated patients into three separate low-, intermediate-, and high-risk groups with 5-year survival rates of 75%, 59%, and 14% respectively. Four of the five characteristics included in the model (uric acid, alkaline phosphatase, LDH, external lymphadenopathy) are probably indicators of high tumor burden, cellular turnover, or aggressive disease. The fifth characteristic (age) is host-related and may reflect the influence of a combination of different measurements of host status, such as organ dysfunction, tolerance to therapy, and other medical illnesses. It is interesting that the annual mortality rate in each risk group remains significantly different throughout the follow-up period. Annual death rates ranged from 2% to 9% for the low-risk, 9% to 23% for the intermediate-risk, and 31% to 42% for the high-risk groups (Fig 7). This supports the concept that CLL heterogeneity is not simply due to detection of patients at different time intervals from onset of disease but that it is truly due to different disease biology. CLL may thus represent a spectrum of diseases with significantly disparate biologic patterns and therapeutic implications. Future studies of cellular markers, cytogenetics, oncogenes, and growth patterns in CLL may help identify these CLL subcategories as different entities that can presently be categorized prognostically on the basis of clinical and biochemical parameters.

The Rai classification is presently widely accepted and confirmed as a useful clinical staging in CLL.² Any proposed new classification, or prognostic model, should provide added value to the Rai staging. Our regression model was superior to the Rai staging in predicting prognosis in the overall

**Fig 7. Risk of death per year for patients in different risk groups.**

population and in the test set. More importantly, it was able to identify, within the various Rai stages, groups of patients whose prognosis was different from that predicted by the Rai classification. For example, 28% of patients with Rai stage 0 could be predicted by the risk model to have a much inferior prognosis and a median survival of only 3.5 years. On the other hand, 20% of patients with Rai 1 or 2 did twice better, and 10% did twice worse than their predicted Rai staging prognosis. It appears, therefore, that the new prognostic model, which incorporates several parameters reflecting tumor burden, disease aggressiveness, and host factors, refines further the resolution of individual prognosis. Thus the use of the regression model alone or combined with the Rai staging should improve our understanding of CLL disease, allow better prediction of individual prognosis, and direct the need and timing of different therapies.

The shortcomings of analysis of prognostic factors in patients with chronic illnesses who receive multiple treatments sequentially are obvious. Changes in the patterns of referral and in therapeutic strategies may also affect the natural course of the disease and modify the relative importance of different prognostic factors.³⁹⁻⁴² In this study there was no change in the referral pattern or in overall survival in time for the total CLL population (Tables 1 and 6). More importantly, there were no significant survival differences even within early and late Rai stages, despite the changes in therapy in the latter category (Table 6). This indicates the stability of the course of CLL disease in time, supporting the validity of the prognostic factors derived.

In summary, our analysis of prognostic factors in CLL identified important clinical and biochemical variables that affected survival and resulted in a regression model dividing patients into three different risk groups. The improved ability to estimate the individual prognosis of patients with CLL should assist in the design and analysis of subsequent clinical trials.

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Functional Differentiation of Normal Human Neutrophils

By Lewis Glasser and Roger L. Fiedlerlein

In the past differentiation of human neutrophils has been defined by morphology, cytochemistry, or surface markers. In our experiments we have sequenced the various events that occur during the functional differentiation of the normal human neutrophil and have also examined some of the functional properties in relationship to surface markers and biochemical events. Granulocytes were obtained from the bone marrow and blood of hematologically normal individuals. Cells were separated into different stages of maturation by their physical properties using counterflow centrifugal elutriation and density gradient separation. Three cell fractions were obtained that were enriched for either immature myeloid cells, band neutrophils, or segmented neutrophils. Since the enriched fractions were not entirely pure, methodologies for functional assays were chosen that allowed cytologic evaluation of the functional

capacity of each cell type. The criteria used to classify the stages of differentiation included both morphology by light microscopy and DNA labeling with tritiated thymidine. Various neutrophilic properties were studied: Fc receptors, complement receptors (CR₁, CR₃), phagocytosis of both live and dead opsonized *Staphylococcus aureus*, microbial killing of *S aureus*, NBT dye reduction after cellular stimulation with endotoxin, and chemotaxis. Our results indicate that the functional properties of the neutrophil appear in a distinct order. The sequence for the functional differentiation of the human neutrophil appears to be the following: Fc receptors → immune phagocytosis → complement receptors → oxygen-independent microbial killing → oxygen-dependent microbial killing → chemotaxis.

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DIFFERENTIATION of human neutrophils is traditionally defined by the morphology of stained cells as seen by light microscopy. Schilling divided developing neutrophils into six morphologic subtypes: myeloblasts, promyelocytes, myelocytes, metamyelocytes, band neutrophils, and segmented neutrophils.¹ Subsequent ultrastructural cytochemical studies showed the sequential acquisition of primary and secondary granules, providing additional criteria for accurately assessing the stages of neutrophilic development.² Although such a classification tends to create an impression of step-like transitions rather than a continuous process, it provides useful and easily understood reference points for keying developmental events in the life history of the neutrophil.

With the development of immunologic techniques and monoclonal antibodies (MoAbs), the sequential appearance of surface markers on the developing neutrophil is now being catalogued.³⁻⁷ In addition to morphologic, enzymatic, and antigenic changes, functional changes also occur during differentiation. The neutrophil becomes more deformable, more adherent to surfaces, more actively forms pseudopods, more avidly ingests particles, and becomes more actively motile.⁸ The maturation sequence of the functional properties of the neutrophil has not yet been determined. Previous studies in our laboratory on leukemic cells suggested that the various functional properties of the neutrophil appear in a distinct order during cell development.⁹ In the present study we have sequenced the various events that occur during the functional differentiation of the normal human neutrophil. Granulocytes were isolated from the bone marrow and blood and separated into different stages of maturation by their physical properties using counterflow centrifugal elutriation and density gradient centrifugation. Three leukocyte fractions were obtained. These were enriched or purified for either segmented neutrophils, band neutrophils, or immature myeloid cells. The enriched fractions were not entirely pure. Thus methodologies were chosen that allowed cytologic evaluation of each stage of differentiation. Phagocytosis, bacterial killing, chemotaxis, nitroblue tetrazolium dye reduction, and opsonic receptors were evaluated. Our results indicate that phagocytosis is acquired early, followed by

nonoxidative microbial killing, and that oxygen-dependent microbial killing and chemotaxis represent the terminal stages of functional differentiation.

MATERIALS AND METHODS

Bone marrow samples. Specimens were obtained from seven paid hematologically normal volunteers of the hospital staff, two hematologically normal individuals undergoing heart surgery, and one patient with Hodgkin's disease. Informed consent was obtained. Not all tests were performed on all specimens. Marrow was aspirated from the iliac crest or sternum. Particles were disrupted by successively passing the sample through 22-G and 25-G needles. Cells were resuspended in 6% hydroxyethyl starch in 0.9% sodium chloride (American McGaw, Irvine CA), allowed to settle at room temperature, and the leukocyte-rich supernatant collected. Segmented neutrophils and eosinophils were removed by Ficoll-Hypaque separation (sp. gr. 1.077) and residual erythroid cells by hypotonic lysis.¹⁰ Nucleated bone marrow cells were then resuspended in elutriation medium consisting of RPMI 1640 and 5% fetal calf serum.

Counterflow centrifugal elutriation. Bone marrow cells were fractionated by centrifugal elutriation using a Beckman J2-21 centrifuge, Je-6B rotor, standard chamber, and Cole-Parmer Masterflex peristaltic pump.¹¹ All procedures were done at 22 °C. The rotor speed was 1740 ± 10 rpm. Fifty-milliliter samples were collected at each flow rate for a total of 16 fractions. Fractions that were similar were combined when necessary to increase the yield. The fractions were further enriched, if necessary, eliminating segmented neutrophils using Ficoll-Hypaque separation. The viability of the cells was determined in each fraction prior to testing using eosin-Y dye exclusion.¹²

Segmented neutrophils were prepared by collecting venous blood in heparin, adding 2 mL of a 6% hydroxyethyl starch solution to 7

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mL of whole blood, harvesting the leukocyte-rich plasma, and eliminating the RBCs by hypotonic lysis. The blood leukocytes were resuspended in elutriation medium and then fractionated using centrifugal elutriation. Monocytes were effectively removed by the elutriation procedure. Conditions were similar to the separation procedure for bone marrow.

Criteria used to classify stages of neutrophil differentiation. Stages of neutrophil differentiation were evaluated by morphologic criteria from smears stained with Romanowsky dyes and by autoradiography after DNA labeling with tritiated thymidine.¹³ Nuclei were considered labeled if five or more grains were counted. The criteria are listed below:

(1) Myeloblasts range from 15 to 20 μm in diameter and have round nuclei with stippled chromatin with one or more nucleoli, a high nuclear/cytoplasmic ratio, and nongranular cytoplasm. They show nuclear labeling with tritiated thymidine.

(2) Promyelocytes differ from myeloblasts by the appearance of azurophilic granules in the cytoplasm.

(3) Neutrophilic myelocytes differ from promyelocytes by the appearance of specific neutrophilic granules. The nuclei show some chromatin condensation, and nucleoli are not as prominent as in the preceding stage. On Romanowsky-stained smears, the cells have round, oval, or flattened nuclei without indentation; however, this criterion was superseded when indented nuclei showed labeling with tritiated thymidine.¹³

(4) Neutrophilic metamyelocytes differ from myelocytes by the appearance of a nuclear indentation and absence of nuclear labeling with tritiated thymidine.

(5) Neutrophilic bands differ from metamyelocytes in that the nuclear indentation is more than half the diameter of a hypothetical circle and the nucleus has parallel sides.

(6) Segmented neutrophils differ from bands in that the nucleus is divided into lobes separated by a thin filament with no visible nuclear chromatin between the two sides.

Fc-IgG receptors. Fc-IgG receptors (FcR) were detected by rosette formation with group O type Rh₁Rh₂ erythrocytes sensitized with human anti CD (Ripley) serum (EA_{hm}).^{14,15} Rosettes of sensitized erythrocytes attached to leukocytes were evaluated in wet preparations and on stained smears prepared with a Larc spinner (Corning Glass Works, Medfield, MA). The percentage of positive cells at each stage of differentiation was evaluated by morphology from the smears. A positive cell was considered to be a leukocyte with one or more attached erythrocytes. Anti-Leu-11b (Becton Dickinson, Mountain View, CA) was also used to study the Fc receptor.¹⁶ Immunogold was used as the detection system (Geometric Data, Wayne, PA). Twenty microliters of 10⁶ leukocytes was mixed with 20 μL of 0.5% paraformaldehyde and 5 μL of anti-Leu-11b. The suspension was incubated at 5 °C for 15 minutes. The paraformaldehyde was necessary in this initial step to prevent phagocytosis of the colloidal gold by the phagocytes. After washing, 20 μL of the colloidal gold reagent was added and incubated at room temperature for 15 minutes. Cells were washed, 5 μL of autologous EDTA plasma was added, and the cells were incubated for 20 minutes at 37 °C. Two microliters of 30% bovine albumin was then added, and push smears or cytocentrifuged slides were prepared and stained. Positive cells have dark blue-black particles visible on the cell surface.

Complement receptors. Complement receptors were assayed with MoAbs (Becton Dickinson): anti-CR₁ (C3b, C4b receptor), anti-CR₃/anti-Leu-15 (C3bi receptor). The method is similar to that described for FcR using MoAbs and immunogold. The classification of the different stages of differentiation was easily evaluated from Romanowsky-stained smears.

Phagocytosis. Neutrophil phagocytosis was evaluated using *S. aureus* (ATCC strain 25933) as the test particle. The bacteria were

opsonized by adding normal pooled human AB serum for 30 minutes at 37 °C. Live bacteria were used to determine the percentage of phagocytic neutrophils, since this results in optimal phagocytosis.¹⁷ Bacteria that were heat killed at 60 °C for one hour were used to evaluate differences in the number of particles ingested per phagocyte to eliminate intracellular bacterial multiplication as a variable. In the reaction mixture the concentration of the specific neutrophilic cell-type being evaluated was 1,000/ μL , the bacteria to leukocyte ratio ranged from 20:1 to 50:1, the final test volume was 0.5 mL, and the suspending medium was pooled human AB serum. The leukocyte-bacterial mixtures of each of the fractions were incubated for one hour at 37 °C, with constant agitation, in polystyrene test tubes. Then the leukocytes were labeled with tritiated thymidine for 60 minutes. Smears were made with a Larc spinner. All smears were stained with Giemsa solution. Five hundred cells at each stage of differentiation, except for myeloblasts and progranulocytes, were evaluated for determining the percentage of phagocytic cells, and 50 phagocytic cells at each stage were evaluated for the number of ingested particles per phagocyte.

Microbial killing. Bacterial killing was assayed by a modification of Cline's technique using *S. aureus*.¹⁸ Mixtures of bacteria with the specified cell type were incubated at a ratio of 20:1 for 30 minutes at 37 °C in a test tube with continuous agitation. After phagocytosis, the cells were washed. A 0.5-mL solution of McCoy's medium with 30% AB serum and tritiated thymidine (New England Nuclear Corp, Boston, 60 to 80 Ci/mmol) was added to 1.0 mL of the cell suspension. The final mixture contained 3 μCi of tritiated thymidine. Cells were incubated for 30 minutes at 37 °C for labeling, and slides were prepared for autoradiography. The total number of intracellular organisms and the number of organisms associated with grains were counted in 50 phagocytic cells of each neutrophilic cell type. The percentage of viable bacteria was calculated from the percentage of bacteria labeled, corrected for 100% labeling.¹⁸

Autoradiography. Cells were labeled with tritiated thymidine of high specific activity (60 to 80 Ci/mmol) for the labeling of both leukocytes and bacteria, as described above. Autoradiography was done as described by Durie and Salmon.¹⁹ Smears were stained through the emulsion with Giemsa solution.

Nitroblue tetrazolium dye reduction. The respiratory burst was evaluated by a modification of the cytochemical technique of Park et al.²⁰ Cells were suspended in 0.5 mL of AB serum to which 0.05 mL of 100 $\mu\text{g/mL}$ of Bacto lipopolysaccharide (Difco, Detroit) was added. A 0.1-mL aliquot of this mixture was added to an equal volume of 0.1% nitroblue tetrazolium (Sigma, St. Louis). A drop of the mixture was added to a glass slide, and the cell suspension was covered with a coverslip that was sealed and then incubated at 37 °C for 30 minutes. In two experiments cells were examined with phase and light microscopy to evaluate both the stage of cellular maturation and dye reduction. At least 200 cells were counted in each fraction. In another experiment we attempted to improve the identification of the stage of cellular differentiation by vital staining with acridine orange (Fisher Scientific, Fair Lawn, NJ). Freshly prepared acridine orange (AO) solution (14.0 mg/100 mL in HBSS, pH 7.2) was added to the cells, mixed, and stained for 45 seconds at room temperature. The AO-stained cells were 98% viable. The cells were then suspended in 0.5 mL of AB serum to which 0.05 mL of endotoxin was added, and the remaining procedure was identical to that described previously. Nitroblue tetrazolium (NBT) dye reduction was evaluated by light microscopy and cell morphology by both light and fluorescent microscopy.

Locomotion. Chemotaxis was evaluated in vitro by the agarose technique.^{21,22} The total inoculum of the desired cell type was 10⁵. The chemotactic well was filled with thawed fresh-frozen AB serum. Interaction of the agarose with the serum generates complement-derived chemotactic factors, eg, C5a. Cells were incubated for five

hours at 37 °C in 5% CO₂ atmosphere with humidity and were stained with Giemsa solution. Chemotaxis was quantitated by counting all the leukocytes of the desired cell type that had migrated out of the well. The cells were identified by light microscopy under oil immersion ($\times 1000$).

Electron microscopy. Leukocytes were fixed in 3% glutaraldehyde made up in 0.1 mol/L phosphate buffer, postfixed in osmium tetroxide, dehydrated in alcohol, and embedded in Spurr's epoxy resin. Sections were cut on a Sorvall MT2-B ultramicrotome and mounted on uncoated 200-mesh grids. A 5% aqueous uranyl acetate solution followed by lead citrate increased contrast. The grids were lightly carbon coated and examined with the Hitachi HU-12 electron microscope. In one experiment, phagocytic myeloid cells were classified ultrastructurally using myeloperoxidase as a marker for primary granules. After phagocytosis cells were fixed in cold 3% glutaraldehyde (pH 7.2), washed, and reacted with diaminobenzidine (1 mg/mL) in 0.05 mol/L Tris buffer containing 0.03% hydrogen peroxide for two hours in a dark room. There was no postfixation in osmium tetroxide or staining with uranyl acetate or lead citrate.

RESULTS

Leukocyte fractions. Neutrophilic leukocytes were separated from the bone marrow or blood on the basis of size and density using gradient-density separation and counterflow centrifugal elutriation, which provided sufficient numbers of cells for functional testing. Mean leukocyte viability of the fractions used for testing ranged from 95% to 100%. We were able to obtain fractions of segmented neutrophils from the blood essentially free of contamination from band neutrophils and fractions of band neutrophils from the bone marrow essentially free of segmented neutrophils. The fraction of immature cells from the bone marrow contained a mean of 60% intermediate (myelocytes and metamyelocytes) neutrophils and 3.9% promyelocytes. It also contained a moderate number of band neutrophils. The percentages of each cell type in each fraction are shown in Table 1. Since the enriched fractions were not entirely pure, techniques for the measurement of neutrophil function were selected that allowed cytologic evaluation.

Fc-IgG receptors. The percentage of EA rosettes in wet preparations and stained smears were in close agreement. In stained smears, sensitized erythrocytes that attached to Fc

Table 1. Differential Counts of Hematopoietic Cells From Bone Marrow and Blood Separated by Centrifugal Elutriation

Cell Type	Fractions*		
	Segmented Neutrophils	Band Neutrophils	Immature Neutrophils
Myeloblast	0	0.9 $\pm$ 0.3	0.6 $\pm$ 0.3
Promyelocyte	0	1.8 $\pm$ 0.6	3.9 $\pm$ 0.8
Myelocyte	0.3 $\pm$ 0.2	9.3 $\pm$ 1.5	35.8 $\pm$ 2.7
Metamyelocyte	0.9 $\pm$ 0.4	24.3 $\pm$ 2.2	23.8 $\pm$ 2.6
Band neutrophil	4.6 $\pm$ 2.2	50.2 $\pm$ 1.9	18.2 $\pm$ 1.8
Segmented neutrophil	86.9 $\pm$ 2.3	4.3 $\pm$ 1.6†	3.1 $\pm$ 1.4‡
Others†	7.3 $\pm$ 1.8	9.2 $\pm$ 1.8	14.6 $\pm$ 2.8

*Mean $\pm$ SEM (n = 9). Results are expressed as a percentage.

†Others (lymphocytes, monocytes, eosinophils, basophils, plasma cells, normoblasts)

‡Contaminating segmented neutrophils were eliminated by density gradient centrifugation.

Table 2. Expression of Fc Receptors (EA_{hu} Rosettes) on Differentiating Neutrophils

Stage	Fc-positive cells %*
Myeloblast	—†
Promyelocyte	32
Myelocyte	42
Metamyelocyte	55
Band neutrophil	78
Segmented neutrophil	82

*Mean values (n = 2 experiments).

†Too few cells seen for evaluation.

receptors on leukocytes were closely applied, frequently molded, and often showed fine hairlike projections extending from the erythrocyte to the leukocyte. The earliest cells, in which Fc receptors were detected, were promyelocytes. Fc-positive cells reached their optimal expression at the band and segmented neutrophil stages (Table 2). Results using the rosette technique differed from those obtained using anti-Leu-11b. In two experiments using the MoAb, only the most mature neutrophils were Leu-11b positive: 82% segmented neutrophils, 40% band neutrophils, 3% neutrophilic metamyelocytes, 0% neutrophilic myelocytes, 0% promyelocytes, 0% myeloblasts.

Complement receptors. Monoclonal antibodies to complement receptors detected CR₁ and CR₃ on neutrophilic leukocytes. These receptors appeared later in development than FcR. Complement receptors were occasionally detected on promyelocytes. CR₃ appeared first and was the dominant receptor in later stages of development. CR₃ was first detected at the promyelocyte stage. A marked increase in expression occurred in cells of the nondividing maturation pool. Thus metamyelocytes had a sixfold increase in positive cells compared with myelocytes. Greater than 90% of band and segmented neutrophils reacted with anti-CR₃ (anti-Leu-15). Results are summarized in Table 3.

Phagocytosis. Even a rare blast had the capacity to phagocytize, and the number of phagocytic cells gradually increased as the cells matured. The phagocytic capacity of immature cells was confirmed by electron microscopy (Fig 1). There was a sharp increase in phagocytic cells as cells entered the nondividing maturation pool. Thus there were more than twice as many phagocytizing metamyelocytes as myelocytes. Peak phagocytosis was reached at the band stage. In two experiments mean values for the percentage of phagocytic cells were: myeloblasts <1%, promyelocytes 10%,

Table 3. Expression of CR₁ and CR₃ on Differentiating Neutrophils

Stage	Anti-CR ₁ %*	Anti-CR ₃ %*
Myeloblast	0	0
Promyelocyte	0	3
Myelocyte	0	10
Metamyelocyte	0	58
Band neutrophil	11	92
Segmented neutrophil	58	94

*Mean values (n = 2 experiments).

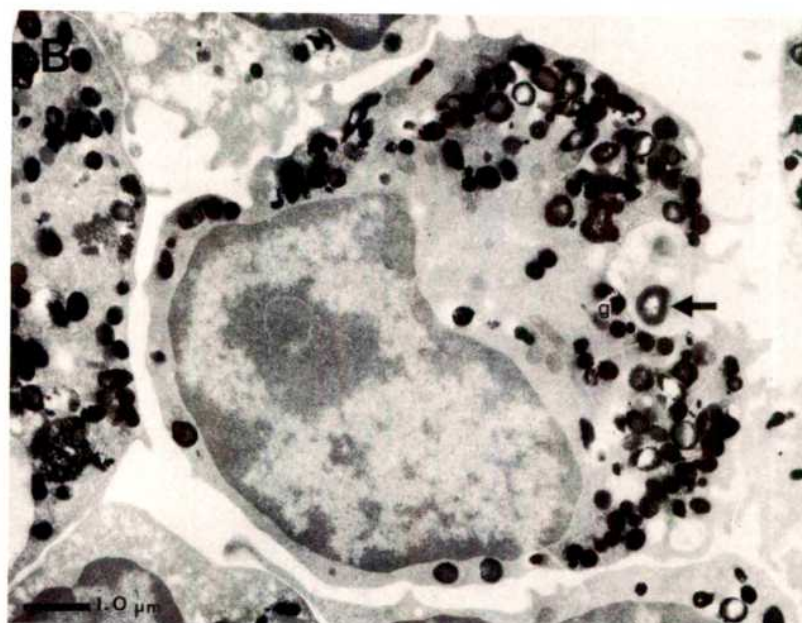
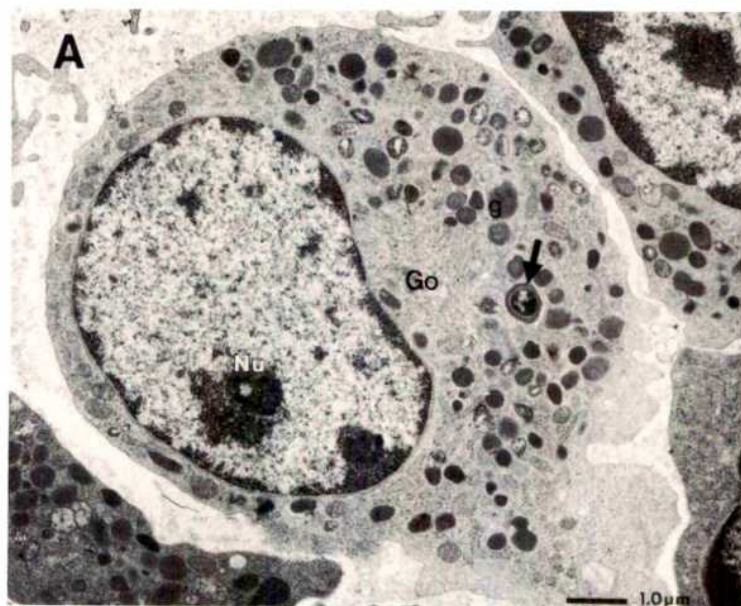


Fig 1. Electron micrographs of granulocyte precursors obtained from the bone marrow of normal subjects. (A) This cell is consistent with a progranulocyte. It has an ovoid nucleus with euchromatin and two nucleoli (Nu). There are numerous electron dense cytoplasmic granules (g). The Golgi (Go) zone is large. Adjacent to the Golgi is a phagocytized bacterium, *Staphylococcus aureus* (arrow). The microorganism is surrounded by an electron-dense material that may represent plasma protein opsonins. No phagolysosome formation is evident. (Uranyl acetate, lead citrate, original magnification $\times 12,400$, current magnification $\times 8,060$.) (B) Immature granulocyte. The cell contains numerous mature primary peroxidase-positive electron-dense granules, has a slightly indented nucleus and prominent nucleolus, and lacks peroxidase in the endoplasmic reticulum. This cell is a "hiatal" cell in transition between a promyelocyte and myelocyte. A bacterium (arrow) has been phagocytized. It is in a vacuole to which numerous peroxidase-positive granules are adherent, forming an incipient phagolysosome. (Diaminobenzidine, original magnification $\times 13,200$; current magnification $\times 8,580$.)

myelocytes 34%, metamyelocytes 84%, band neutrophils 99%, and segmented neutrophils 99%. The phagocytic capacity (ie, bacteria/phagocyte) of each cell type showed the same trend as percent phagocytosis. Too few phagocytic cells were seen at the myeloblast and promyelocyte stage for a meaningful comparison with other stages of maturation. There was a marked increase of phagocytic capacity as cells entered the nondividing maturation pool. Mean values for two experiments showed that myelocytes ingested seven bacteria/cell, metamyelocytes 14/cell, band neutrophils 36/cell, and segmented neutrophils 38/cell.

Stimulated NBT dye reduction. The techniques used in these experiments allowed us to identify each morphological stage of differentiation showing nitroblue tetrazolium dye reduction. The results were striking. Only band and segmented neutrophils showed a "respiratory burst," as evalu-

ated with the cytochemical technique. Also there was a significant difference in NBT reduction between band and segmented neutrophils ($p < 0.05$). Mean values in three experiments were: myeloblasts 0%, promyelocytes 0%, intermediate neutrophils (myelocytes and metamyelocytes) 1%, band neutrophils 57%, and segmented neutrophils 98%.

Microbial killing. Two normal cell donors were tested to evaluate bacterial killing. Both the percentage of bacteria killed by phagocytic cells of each cell type and the total number of bacteria killed by equivalent numbers of leukocytes of each cell type were determined. Two few phagocytic promyelocytes were seen for a statistical comparison of bacterial killing with other cell types; however, the few phagocytic promyelocytes seen showed only modest killing, and two thirds of the bacteria survived intracellularly. Since promyelocytes did not reduce nitroblue tetrazolium, all kill-

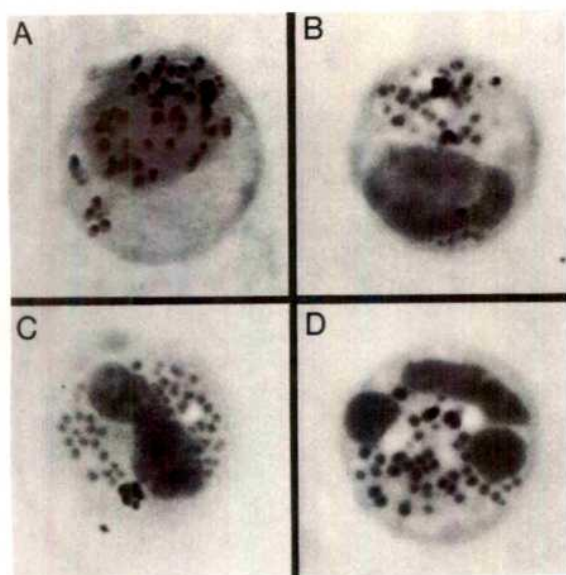


Fig 2. Bactericidal activity of developing neutrophils. Following ingestion, bacteria-leukocyte mixtures were exposed to tritiated thymidine. Autoradiographs show a progressive increase in bactericidal activity with progressive differentiation of the neutrophil. (A) Myelocyte: Several grains on the nucleus indicate active incorporation of thymidine. The cytoplasm shows several labeled bacteria. (B) Metamyelocyte: The nucleus is not labeled. There are numerous ingested bacteria, some showing labeling. (C) Band neutrophil: Numerous intracellular dead (unlabeled) bacteria and a few labeled bacteria. (D) Segmented neutrophil: Numerous intracellular bacteria with only a single labeled organism. Original magnification $\times 500$.

ing was attributed to oxygen-independent mechanisms. Each successive stage of neutrophil differentiation showed increased bacterial killing with decreased numbers of viable intracellular bacteria (Fig 2). Myelocytes were poor killers, and as many as 48% of the ingested bacteria were viable. As in the promyelocyte, no mechanism for oxidative killing was demonstrated. Metamyelocytes also did not reduce NBT but killed a substantial number of ingested organisms by oxygen-independent mechanisms. A mean of only 11% of ingested organisms were viable after interacting with metamyelocytes. This cell type demonstrates the competence of oxygen-independent killing mechanisms. Band neutrophils killed more microorganisms both relatively and quantitatively, containing a mean of 8% viable intracellular bacteria. This cell type reduced NBT. Thus killing involved both oxygen-independent and oxygen-dependent mechanisms. Segmented

neutrophils were the most effective bactericidal cells. Only 5% of the bacteria were viable after ingestion. As shown in Table 4, differences in the bactericidal capacity of each cell type are much greater when the total number of bacteria killed by equivalent numbers of leukocytes are compared.

Chemotaxis. Chemotaxis was a later manifestation of functional maturity. Essentially no chemotaxis was observed except in band and segmented neutrophils. Band neutrophils showed approximately half the activity of segmented neutrophils. In two experiments the mean chemotactic scores for the three fractions analyzed were intermediate neutrophils (myelocytes, metamyelocytes) 6, band neutrophils 424, and segmented neutrophils 900 (Fig 3).

DISCUSSION

The sequence of development of the functional properties of the neutrophil during differentiation has not previously been determined. Neutrophils must have the biological functions needed to eradicate microbes. In the inflammatory process a distinct sequence of events occurs. Locomotion, diapedesis, and chemotaxis precede phagocytosis. Ultimately the internal killing mechanisms of the cell destroy the microbe. During differentiation the functional properties of the neutrophil are acquired sequentially. Our data show that phagocytosis is acquired prior to the functions, followed by oxygen-independent microbial killing, whereas both oxygen-dependent microbial killing and chemotaxis are late manifestations of functional differentiation. According to our data the relative activity of each neutrophilic cell type for each function is shown in Fig 4.

Fc and complement receptors are acquired early in differentiation.⁴ Both are important in immune phagocytosis. Interaction of Fc receptors with opsonins trigger second messages, promoting both phagocytosis and superoxide generation.²³ Our results show that Fc receptors appear at least as early as the promyelocyte stage. Results of the rosette technique using sensitized erythrocytes differed from those of the MoAb Leu-11b. The former must be considered the reference method for the qualitative detection of Fc receptors. There are several possible explanations for the differences. The absence of Leu-11b on immature granulocytes may indicate that further molecular modeling of Fc receptors occurs at the band neutrophil stage. It is also possible that the antigenic determinant Leu-11b is not part of the Fc receptor even though anti-Leu-11b inhibits the function of the receptor. Another explanation is that the FcR epitope detected by anti-Leu-11b is not critical for the binding of IgG-sensitized erythrocytes. In murine neutrophils it has been proposed that Fc receptors appear on the cell membrane before the cellular acquisition of phagocytosis.²⁴ Murine myelocytes were the earliest cells to acquire Fc receptors; however, only segmented neutrophils or neutrophils with O-ring nuclei were phagocytic. Altman and Stossel studied phagocytosis in developing human neutrophils, and they did not observe phagocytosis by promyelocytes.²⁵ In our experiments human neutrophils showed phagocytosis as early as the promyelocyte stage, and phagocytic capacity lagged slightly behind but paralleled the acquisition of Fc receptors. We used enriched fractions of bone marrow rather than a mixture of

Table 4. Neutrophil Differentiation and Bacterial* Killing

Stage	Bacteria Killed/100 Cells		Mean Relative Activity %
	Exp #1	Exp #2	
Myeloblast	0	0	0
Promyelocyte	24	0	<1
Myelocyte	282	54	4
Metamyelocyte	1969	670	28
Band neutrophil	3454	3100	72
Segmented neutrophil	4004	5450	100

**Staphylococcus aureus*.

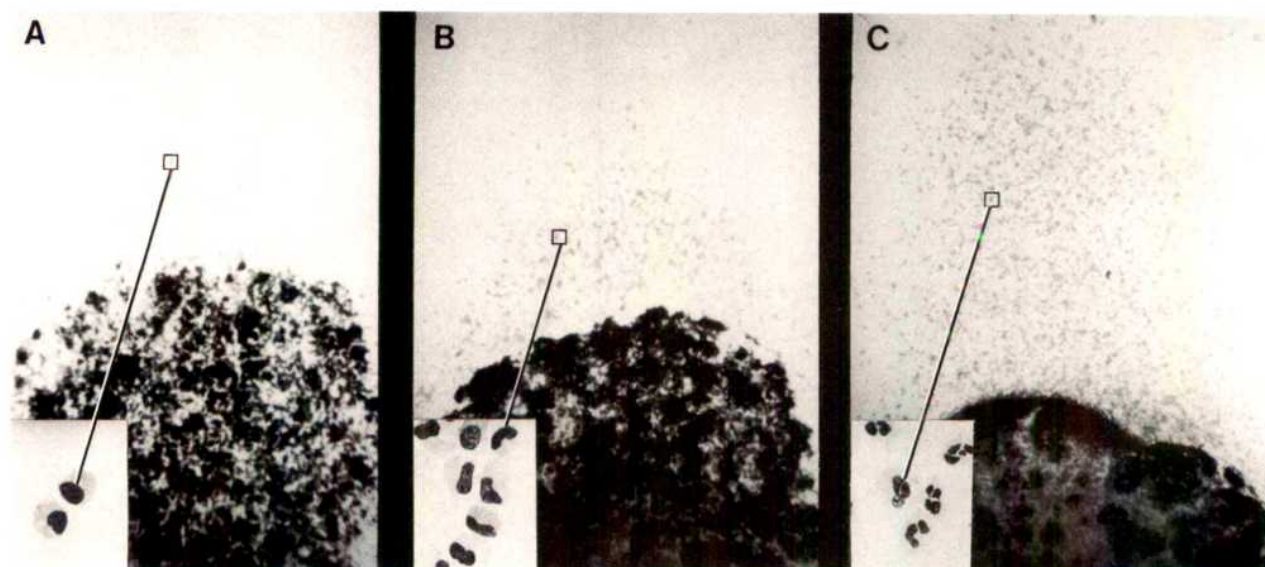


Fig 3. Neutrophil migration under agarose. (A) Myelocyte/metamyelocyte fraction. The well (bottom) contains immature granulocytes that show little migration. A rare myelocyte and metamyelocyte (inset) are seen migrating toward the chemotactic attractant. (B) Band neutrophil fraction. A moderate number of band neutrophils migrate toward the chemotactic attractant. (C) Segmented neutrophils. Numerous segmented neutrophils migrate toward the chemotactic attractant.

all bone marrow leukocytes. This enabled us to standardize the ratios for each cell type and to diminish the competitive effect of more actively phagocytic cells, thereby allowing leukocytes such as promyelocytes to express their full phagocytic potential. Enriched fractions may simply allow more promyelocytes to be evaluated experimentally. We also used a different particle, live bacteria rather than opsonized albumin or lipopolysaccharide-coated paraffin oil droplets. The type of particle and the method of preparation of the particle are critical. For example, in our experiments we noted that promyelocytes phagocytized opsonized live bacteria more readily than opsonized dead bacteria. This is consistent with the observation that human segmented neutrophils distinguish between viable and nonviable bacteria and preferentially ingest live organisms.¹⁷ The best evidence in humans that Fc receptors are acquired by granulocytes

prior to the capacity for phagocytosis comes from studies with human myeloid leukemic cell strains.⁹ Whereas some leukemic strains show prominent EA_{hu} rosettes and little or no phagocytosis, no Fc receptor-negative myeloid-leukemic strains have shown phagocytosis. Thus human leukemic cells clearly demonstrate that the acquisition of Fc receptors precedes phagocytosis.

In addition to FcR, complement receptors also promote phagocytosis. Ligands derived from the third component of complement (C3) are the principal opsonic forms of complement. There are two complement receptors on neutrophils that react with C3 opsonins: CR_1 and CR_3 . These receptors react synergistically with Fc receptors to promote phagocytosis.^{23,26} They appear at a later stage of development than Fc receptors and phagocytosis and are not overtly expressed until the myelocyte/metamyelocyte stage. Once complement receptors are acquired, phagocytosis is enhanced. It is teleologically satisfying that the receptor for the predominant opsonic species C3bi appears prior to other complement receptors.

The next function acquired by differentiating neutrophils was microbial killing. The labeling technique coupled with the NBT test can be used to assess the contribution of both oxygen-independent and oxygen-dependent bactericidal mechanisms. It is well established that the biochemical basis of NBT reduction in normal human neutrophils is due to superoxide generation and that cytochemical NBT reduction is an expression of the respiratory burst.²⁷ Phagocytic promyelocytes killed few bacteria. Promyelocytes have no oxidative killing; however, it also appears that their oxygen-independent bactericidal mechanisms are inoperative. Promyelocytes have primary granules that contain lysosomal enzymes, lysozyme, and cationic bactericidal proteins. Thus the armamentarium for oxygen-independent killing is present. It is possible that suboptimal phagolysosome formation

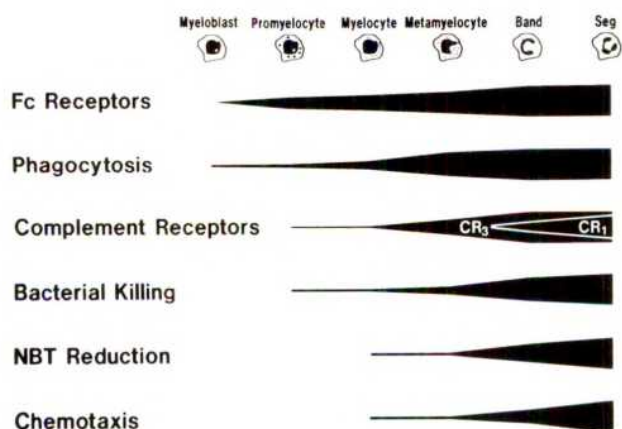


Fig 4. Schematic representation of the relative activity of neutrophil functions at various stages of neutrophil differentiation.

or absence of secondary granules, which contain bactericidal substances such as lactoferrin, accounts for impaired nonoxidative killing. Like promyelocytes, neither myelocytes nor metamyelocytes showed a respiratory burst; however, both cell types killed bacteria. The importance of neutrophilic nonoxidative microbial killing has been thoroughly reviewed by Spitznagel.²⁸

Oxygen-dependent bacterial killing is a late manifestation of functional differentiation. Only band and segmented neutrophils showed a substantial respiratory burst. Segmented neutrophils had significantly more activity than band neutrophils. These results are similar to the observations of Zakhireh and Root.²⁹ Since oxygen-independent and oxygen-dependent antimicrobial mechanisms are synergistic and overlapping, it may not be possible to assess their relative roles in band and segmented neutrophils.

Chemotaxis is also a late manifestation of functional differentiation. Previous studies on immature myeloid leukemic cells indicated that random motility was expressed in the absence of chemotaxis.⁹ Further modulation of the cell is necessary for chemotaxis. This may relate to the molecular modeling of C5a receptors, the translation of chemotactic stimuli, or the cytoskeleton.^{30,31} Studies using the human promyelocytic leukemia HL60 cell line as a model of myeloid

differentiation show that the binding of chemotactic factors to receptor sites alone is not sufficient for chemotaxis.³² In our study chemotaxis was rarely observed except in band and segmented neutrophils. The latter had twice the activity of band neutrophils. Thus chemotaxis is expressed late and represents a terminal stage of functional differentiation.

We have studied a fundamental aspect of leukocyte differentiation, neutrophil functional differentiation. According to these studies we propose the following sequence for the functional differentiation of the neutrophil: Fc receptors → immune phagocytosis → complement receptors → oxygen-independent microbial killing → oxygen-dependent microbial killing → chemotaxis.

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F-Actin Content of Neonate and Adult Neutrophils

By Ann Hilmo and Thomas H. Howard

We utilized flow cytometric analysis of NBDphalloidin-stained cells to measure F-actin content, expressed as fluorescent channel numbers, and we compared the microfilamentous cytoskeletal organization in neutrophils from healthy neonates (36 to 38 weeks gestational age) and adults. Basal F-actin content in neonate cord blood neutrophils is higher than that of adults. The elevation is intrinsic to the cell and not related to parturition because basal F-actin content of neonatal cells obtained by venipuncture (days 1 to 8 of age) is also elevated (38 ± 10 , $N = 15$) when compared to adults (21 ± 7.0 , $N = 27$). The rate of N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced actin polymerization is similar in adult and neonatal neutrophils and is maximal by 30 to 45 seconds at 25 °C. Adult, neonatal cord, and neonatal venipuncture neutrophils increase F-actin content to a similar extent following 0.5 $\mu\text{mol/L}$ fMLP activation (52 ± 18 , $N = 27$; 58.7 ± 18 , $N =$

18 ; 51.5 ± 7.0 , $N = 15$, respectively). However, the relative increase in F-actin content following fMLP activation is much greater in adult (2.37-fold) than neonatal neutrophils (1.28-fold). This difference is due to the elevated basal F-actin content of neonatal cells. Comparison of distribution of F-actin content among basal, neonatal neutrophils reveals two subpopulations of neutrophils with respect to F-actin content—approximately 25% with F-actin content similar to that of adult neutrophils and 75% with F-actin content greater than that of adult cells. Following fMLP activation, the subpopulations disappear. The results suggest that abnormalities in microfilamentous cytoskeletal organization of neonatal cells may, in part, be responsible for decreased chemotactic response of neonatal neutrophils.

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BACTERIAL INFECTION is a leading cause of mortality among newborns in the United States.¹ Increased susceptibility of the neonate to bacterial infection is due in part to the immaturity of both the cellular and the humoral immune system.² Defective mobilization of neutrophils into inflammatory sites was one of the earliest cellular abnormalities documented in neonates.³ An *in vitro* corollary of the ineffective mobilization of neutrophils is decreased chemotactic responsiveness of the neonatal neutrophil. Numerous investigators demonstrated decreased chemotactic response of neonatal neutrophils as compared to adult neutrophils.³⁻⁷ Others have suggested that neutrophils from neonates are defective in random locomotion, and critical studies that analyzed both rate and orientational elements of the chemotactic response show that both are defective in neonatal neutrophils as compared to adult neutrophils.⁵

The search for an explanation of the decreased motility of neonatal neutrophils has recently focused on the cytoskeleton of the cell. Studies by Cheung and Miller⁸ showed that the neonatal neutrophil is less deformable than the adult neutrophil. Since cellular deformability is a characteristic of the membrane and the cytoskeleton and since evidence indicates that the orientational element of neutrophil locomotion requires an intact microtubular cytoskeleton,⁹ Anderson and co-workers studied the microtubular cytoskeleton of the neonatal neutrophils.¹⁰ Their studies show that when compared to adult neutrophils, neonatal cells contain fewer, shorter, and more disoriented microtubules than adult cells. These studies suggest that the defective orientational response of neonatal neutrophils may be related to defective organization of the microtubular cytoskeleton. To date the force-generating element of the cell, the microfilamentous cytoskeleton, has not been examined in the neonatal neutrophil.

The molecular composition of the microfilamentous cytoskeleton in phagocytes is clearly described by Southwick and Stossel.¹¹ The major component of this cytoskeleton is actin. Actin is a 43-kD globular protein that can condense to form filaments called F-actin. F-actin can serve either a force-generating role by interacting with myosin or a structural

role through crosslinks formed with other structural proteins like actin-binding protein.¹² Until recently quantification of the mass of F-actin in cells was difficult and required large numbers of neutrophils. Our laboratory recently described a simple, quantitative method for measuring F-actin content. The method is based upon flow cytometric analysis of neutrophils stained with NBDphalloidin—a fluorescent probe that binds specifically to F-actin.^{13,14}

In the studies presented we quantify the F-actin content and compare the microfilamentous cytoskeletal organization of neutrophils from neonates and adults. Our results show that the basal F-actin content of neonatal neutrophils is significantly higher than that of adult neutrophils and that chemotactic peptide-induced actin polymerization is significantly less in neonate than in adult neutrophils. Also, unlike adult neutrophils, neonatal neutrophils are composed of two subpopulations of cells with respect to basal F-actin content. Our results suggest that differences in the microfilamentous cytoskeleton in neonatal and adult neutrophils may relate to the differences in motility and chemotactic responsiveness of neonate and adult neutrophils.

MATERIALS AND METHODS

Preparation of leukocytes. Cord blood was obtained after delivery of healthy term neonates (gestational age 37 to 42 weeks by Dubowitz). After informed consent was obtained, venous blood was

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collected from healthy adults and healthy term neonates (gestational age 36 to 42 weeks by Dubowitz) between 1 and 8 days of age. Whole blood was drawn into plastic syringes with EDTA anticoagulant. Samples were centrifuged in plastic tubes ($900 \text{ g} \times 3 \text{ minutes}$) with a Beckman (Norcross, GA) TJ6 centrifuge. Plasma was removed and cells were suspended in phosphate buffered saline (PBS) pH 7.15 and centrifuged ($1,800 \text{ g} \times 10 \text{ minutes}$). Supernatant was discarded, and the buffy coat, which contains mixed leukocytes, was aspirated and transferred into plastic tubes. Red cells were hypotonically lysed in distilled water for 45 seconds and washed once in PBS. Leukocytes were resuspended in Hanks'/HEPES balanced salt solution¹³ at a concentration of 4×10^6 cells/mL. All steps of cell preparation were completed at 22°C .

Assay of F-actin content. The leukocyte suspension (0.5 mL) was warmed to 25°C . Samples were exposed to 0.5 mL of either dimethyl sulfoxide (DMSO) (0.01 vol % final concentration) or the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma, St. Louis; 5×10^{-7} mol/L final concentration) for the indicated period of time. The fMLP was dissolved in DMSO. After indicated period of time with DMSO or fMLP, cells were fixed with 3.7% formalin for 15 minutes at 25°C , stored overnight at 4°C , and then permeabilized with lysophosphatidylcholine, stained with NBDphalloidin, and analyzed by fluorescence-activated cell sorter. Time from collection of blood to fixation of cells never exceeded five hours.

Quantification of F-actin. Formalin-fixed cells from buffy coat cells were permeabilized with lysophosphatidylcholine and stained with NBDphalloidin as previously described.¹⁴ The sample was filtered with a $50\text{-}\mu\text{m}^2$ nylon mesh and analyzed on a Coulter C flow cytometer. Mixed leukocytes were analyzed for forward light scatter and 90° light scatter to generate a dot plot. As previously described for permeabilized cells,^{15,16} neutrophils are the group of leukocytes on the dot plot with greatest 90° and forward light scatter. Sorting of cells in this gated population revealed $>93\%$ neutrophils on samples from neonates and adults. Cytospin preparations of the gated cells included only bands and segmented forms in both adults and neonates as previously described.^{15,16} The relative proportions of bands and segmented forms among neutrophilic cells from both sources were similar (adults $N = 8$; bands $7.3 \pm 5\%$, the remainder segmented; neonates $n = 8$, bands 9.0 ± 4.3 , the remainder segmented; a rare metamyelocyte in both cases).

After setting the gate for neutrophil population, the log- and linear-scaled fluorescence histograms from 10,000 neutrophils were determined (Argon Laser 5W, excitation at 488 nm, and emission at ≥ 520 nm with a long-pass filter). Fluorescence gain and PMT voltage were identical for all samples. The Coulter C flow cytometer yields values that include mean fluorescence channel number for the 10,000 cell population. F-actin content is expressed as mean fluorescence channel number from linear-scaled histograms. Basal F-actin is defined as mean fluorescence channel number of cells exposed to DMSO for 60 seconds and is identical to cells exposed to buffer for 60 seconds. Neither basal nor fMLP-activated F-actin content was affected by mode of delivery, presence or absence of labor, or expiration of time (one to three hours) before separation of leukocytes.

For experiments in which cumulative, normalized histograms are shown, three-decade log fluorescence histograms were recorded concurrent with the linear histograms. In each case the log fluorescence histograms included all cells observed on the linear histogram, and no additional outliers were present. The peak fluorescence channel always occupied a position approximately two thirds of the way through the second decade of the three-decade scale. The data was normalized by tracing log fluorescence histograms of samples onto tracing paper, marking the horizontal axis at 0.5-cm intervals, and dividing the histograms into constituent portions by placing

vertical lines at 0.5-cm intervals. Then, the total area of each histogram was determined and assigned a value of 100%. Next, the area of each constituent portion of each histogram was determined and expressed as a percentage of the whole histogram area. The percentage of histogram area in a 0.5-cm interval reflects the percentage of cells in that interval. The data on several histograms were cumulatively expressed by determining the mean percent ± 1 SD of cells in the constituent portions of N histograms. The areas were measured in triplicate with a GP-650 sonic digitizer from Science Accessories (Highland, NY).

RESULTS

Comparison of basal F-actin content. The basal F-actin content of neonate and adult neutrophils was compared by flow cytometric analysis as described in "Methods." Briefly, subgroups of NBDphalloidin-stained buffy coat cells are characterized by forward and 90° light scatter, gated for neutrophils, and the F-actin content expressed as the mean fluorescence channel number as determined from the linear-scaled histogram of 10,000 neutrophils. The mean fluorescence channel number is the mean for the entire population regardless of whether subpopulations are present or absent.

As shown in Fig 1, the basal F-actin content of neonatal cord blood neutrophils (35.8 ± 15 [$N = 18$]) is significantly higher ($P < 0.001$) than that of adult neutrophils (21.0 ± 7.0 [$N = 27$]). Basal F-actin content of neonate neutrophils obtained by venipuncture is similar to that of cord blood neutrophils (38 ± 10 [$N = 15$]) and higher than adult cells ($P < 0.001$). This result indicates that the elevated basal F-actin content is characteristic of neonate cells and is not a transient abnormality related to parturition. Basal F-actin content of cord samples was similar for neonates delivered vaginally or by caesarean section and was not affected by anesthesia (local v spinal) utilized (data not shown).

Comparison of fMLP-induced actin polymerization. Exposure of adult neutrophils to chemotactic stimuli, such as fMLP, results in rapid polymerization of actin at 25°C . Maximal F-actin content is noted 45 to 60 seconds after activation with fMLP concentrations greater than 10^{-8}

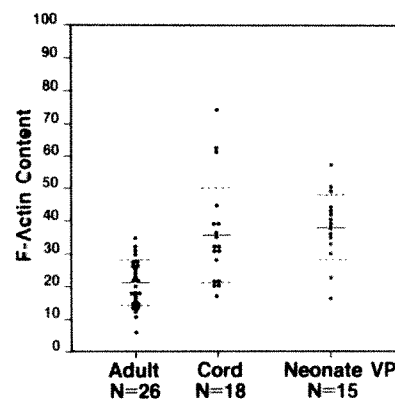


Fig 1. Comparison of basal F-actin content in adult and neonatal neutrophils. Shown are the mean F-actin content ± 1 SD from N number of samples of adult, neonatal cord, and neonatal venipuncture (VP) neutrophils. F-actin content is expressed as mean fluorescence channel number determined by flow cytometric analysis of NBDphalloidin-stained cells. Each dot is one individual.

mol/L.¹⁴ The rapid initial polymerization is followed by a slower depolymerization during five to ten minutes.^{14,17} We compared the fMLP-induced polymerization of actin in neonate and adult neutrophils. Results are shown in Fig 2. Activation of neonatal and adult neutrophils with 0.5 μ mol/L fMLP induces actin polymerization. The time course and rate of fMLP-induced change in F-actin content are similar for adult and cord neutrophils. Both are maximal at ≥ 30 seconds. However, greater variability of polymerization in neonate cells is reflected in the larger standard deviations for neonate than adult cells.

Comparison of F-actin content of neonate and adult neutrophils exposed to fMLP for 60 seconds shows that the maximal F-actin content of fMLP-activated neutrophils from adults (52 ± 18 [N = 27]), neonatal cord blood (58.7 ± 18 [N = 18]), and neonatal venipuncture (51.5 ± 7 [N = 15]) is similar. Since maximal extent of fMLP-induced actin polymerization is similar for adult and neonate neutrophils and since the basal F-actin content of neonate neutrophils is greater than that of adult cells, the fMLP-induced change in relative F-actin content is more heterogeneous but significantly less in neonatal neutrophils (adult 2.76 ± 0.67 , N = 24; cord 1.71 ± 0.59 , N = 17 [$P < 0.001$]; neonate VP 1.28 ± 0.24 , N = 14 [$P < 0.001$]; see Fig 3).

Evidence for subpopulations of neonatal neutrophils. In addition to yielding the mean F-actin content for a population of neutrophils, flow cytometric analysis of NBDphalloidin-stained neutrophils generates a fluorescence histogram that depicts the distribution of F-actin content among constituent cells of a population.^{13,17} In general, the values for F-actin content among 10,000 adult neutrophils are more homogeneously distributed than those from neonates. Values in neonatal neutrophils are broadly spread, and in most cases histograms reveal the presence of two populations of cells with respect to F-actin content. One population is adultlike, the other population has elevated F-actin content. The relative proportions of cells with adultlike F-actin content are variable from one neonate to another. Following fMLP-activation, the F-actin content among neonate and adult

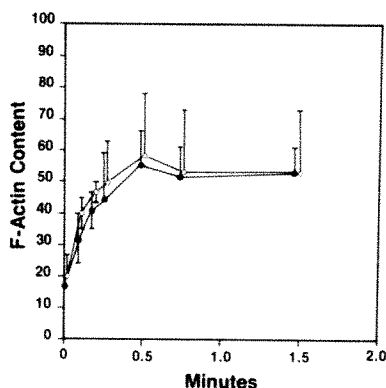


Fig 2. Comparison of fMLP-induced actin polymerization in adult and neonatal neutrophils. Shown are F-actin content at indicated time after exposure of adult (●—●) and neonatal (○—○) neutrophils to 0.5 μ mol/L fMLP. Values are mean $\pm$ 1 SD from five trials.

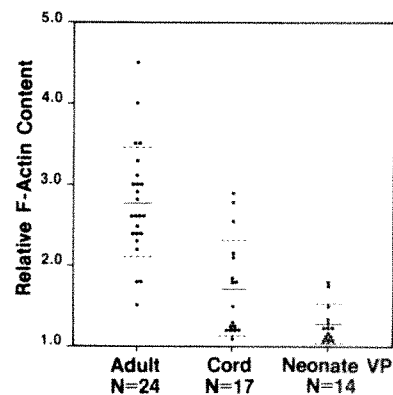


Fig 3. Comparison of fMLP-induced increase in relative F-actin content in adult and neonatal neutrophils. Relative F-actin content is ratio of F-actin content at 60 seconds after 0.5 μ mol/L fMLP/basal F-actin content. Shown are mean relative F-actin content $\pm$ 1 SD from N number of samples of adult, neonatal cord, neonatal venipuncture (VP) neutrophils. Each dot is one individual.

neutrophils is similar, and both are described by more normal distributions.

Comparisons of normalized histograms from larger numbers of adult, neonate cord, and neonate venipuncture samples confirm the presence of subpopulations of neonatal neutrophils. Figure 4 shows the comparison of normalized, log-scale fluorescence histograms of F-actin content in neutrophils from ten adults, 14 neonate cord, and 15 neonate venipuncture samples. Nonactivated neonatal neutrophils from cord or venipuncture samples contain two subpopulations of neutrophils. In contrast, adult neutrophils are represented as a single, normal distribution of neutrophils with respect to F-actin content. On average, approximately 25% of neonate neutrophils have a basal F-actin content similar to

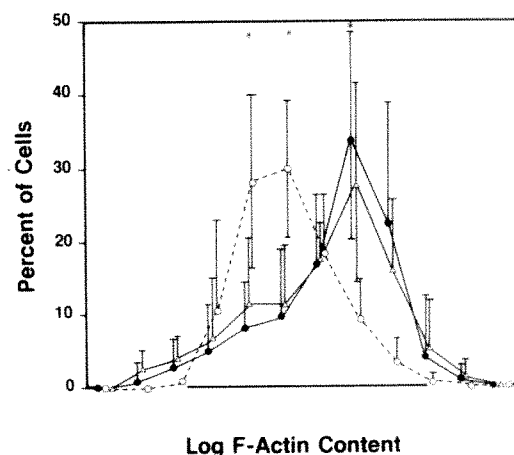


Fig 4. Distribution of basal F-actin content among adult and neonatal neutrophils: cumulative, normalized data. Histograms from ten adults (○—○), 14 neonatal cord (Δ — Δ), and 15 neonatal venipuncture (●—●) samples were normalized as described in "Materials and Methods." Shown are mean percentage $\pm$ 1 SD of neutrophils with F-actin content at 0.5-cm intervals along the log-F-actin histogram. Values are statistically significantly different from adult values at the indicated (*) points.

that of adult neutrophils. The F-actin content of the remaining 75% of neonatal cells is significantly higher than that of adult cells. However, note that in individual patients the relative proportion of cells with elevated basal F-actin content is highly variable (range 48% to 95%, mean 75%).

Comparison of normalized histograms for F-actin content following fMLP activation shows that maximal F-actin content in adult and neonatal neutrophils is similar and more normally distributed at values slightly higher than those of basal adult cells and either of the subpopulations of neonatal neutrophils (Fig 5). Of interest during fMLP-induced actin polymerization, the histograms for both adult and neonate cells broaden within five seconds of fMLP addition. At no time do adult cells contain two distinct subpopulations, and subpopulations among neonatal cells are only apparent in nonactivated cells (data not shown). This result suggests, but does not prove, that the subgroups of neonate cells differ only in the extent of polymerization in the basal cell and do not differ in their kinetics of polymerization.

DISCUSSION

When compared to adult neutrophils, motility of neutrophils from neonates is abnormal. The abnormalities include defects in basal locomotion,^{5,6} chemokinesis,^{5,6} chemotaxis,³⁻⁷ orientational response,¹⁰ concanavalin A (Con A) capping,^{18,19} and abnormally decreased elasticity⁸ of neonatal cells. Anderson et al¹⁰ showed that the microtubular cytoskeleton of neonatal neutrophils is significantly different from that of adult neutrophils. In the presence of chemotactic factors, adult and neonatal neutrophils increase the number, length, and mass of microtubules.¹⁰ However, under similar conditions of activation, neonatal neutrophils contain fewer microtubules of shorter length than do adult neutrophils. Anderson¹⁰ suggests that the differences in microtubu-

lar organization may underlie the known defects in motility of neonatal neutrophils. However, organization of the microfilamentous cytoskeleton in neonatal neutrophils has not been studied. This report quantitatively describes the organization of microfilamentous cytoskeleton in neonatal neutrophils.

In these studies we compare the basal and fMLP-activated microfilamentous cytoskeletons of neonatal and adult neutrophils as reflected by the filamentous actin (F-actin) content of cells. The mean F-actin content and the distribution of F-actin content among neutrophils is determined by flow cytometric analysis of fixed, NBDphalloidin-stained neutrophils.^{13,14} The techniques utilized are readily applicable to studying neonates because they require relatively small volumes of blood. The results show that (1) basal F-actin content of neonatal neutrophils is greater than that of adult neutrophils, (2) the rate of fMLP-induced actin polymerization and maximal extent of actin polymerization in neonatal and adult cells is similar, and (3) with respect to basal F-actin content, neonatal neutrophils are composed of two subpopulations of cells. The results suggest that abnormalities of microfilamentous cytoskeleton in combination with abnormality of microtubular cytoskeleton may underlie the defective motility of neonatal neutrophils.

The basal F-actin content of neonatal neutrophils is significantly greater than that of adult neutrophils. The elevation in basal F-actin content is observed in both cord neutrophils and neutrophils obtained by venipuncture of neonates aged 1 to 8 days. No obvious correlation with mode of delivery, caesarean v vaginal, was noted, and results were reproducible during six months of studies. The differences in basal F-actin content of neonatal and adult neutrophils do not reflect a difference in the ability of their actin to polymerize because the fMLP-induced rates of actin polymerization are similar. Finally, differences in basal F-actin content cannot be explained by different amounts of polymerizable actin in neonatal and adult cells because the maximum extent of actin polymerization is similar for cells from both sources.

The major cytoskeletal consequence of the elevated basal F-actin in neonatal cells is that the magnitude of the chemotactic peptide-induced increase in F-actin content of neonatal cells (1.28-fold $\pm$ 0.24-fold) is significantly less than for adult neutrophils (2.76-fold $\pm$ 0.67-fold). The major consequence of an elevated basal F-actin to motility is less clear. Although F-actin content in fMLP-activated neutrophils correlates with the rate of cellular locomotion under some conditions,¹³ direct correlation of alterations in F-actin content of neonatal cells with the reported defects in locomotion is, at present, impossible. Two points, however, are worthy of note. First, the decreased deformability of neonatal neutrophils that is implied by motility studies in Boyden chambers of varied pore size⁶ and documented by elastimetry⁸ may relate to the increased basal F-actin content of neonatal neutrophils. Logically, the increased amounts of rigid F-actin polymers would produce increased viscosity of cytosol just as F-actin increases the viscosity of aqueous solutions. Secondly, the most consistent, reproducible defects in the motility of neonatal neutrophils—defects in chemotaxis and chemokinesis—are only apparent after

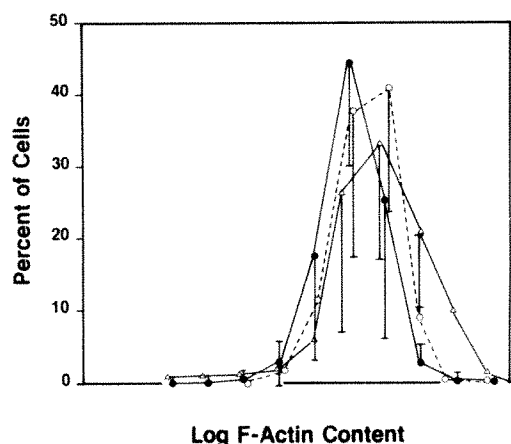


Fig 5. Comparison of fMLP-activated F-actin content among adult and neonatal neutrophils: cumulative, normalized data. Histograms from ten adults (○---○), 14 neonatal cord (▲—▲), and 15 neonatal venipuncture (●—●) samples exposed to 0.5 μ mol/L fMLP for 60 seconds are shown and normalized as described in "Materials and Methods." Shown are mean percentage of $\pm$ 1 SD of neutrophils with F-actin content at 0.5-cm intervals along the log-F-actin histogram. No values are statistically significantly different.

the cell is exposed to chemotactic factors like fMLP. Since elevated basal F-actin limits the magnitude of fMLP-induced change in F-actin content, it is logical that the decreased range of cytoskeletal responsiveness to fMLP may translate into decreased chemotaxis and chemokinesis. Obviously proof of these hypotheses awaits detailed correlation of F-actin content and distribution with chemokinesis and chemotaxis of cells.

The elevated basal F-actin content of neonatal neutrophils should not be misinterpreted as indicating that the actin in neonatal neutrophils differs in polymerizability from the actin of adult neutrophils. Historically the structure and physicochemical behavior of actin is highly conserved in species as disparate as unicellular amoeba and man, despite the occurrence of isotypic variants.¹² Experimentally, as shown here, the gross kinetics of fMLP-induced actin polymerization in adult and neonatal cells is similar. A more tenable explanation for the elevated basal F-actin content of neonatal neutrophils is to assume that in our studies F-actin content simply reflects the state of neutrophil activation and that neonatal neutrophils are more sensitive to activation by undefined factors that are either released during our assays or present during parturition and for one to eight days postnatally. Some experimental data on oxidative metabolism of neutrophils suggest such may be the case.²⁰ Document-

tation of this point will require further studies on F-actin content and oxidative metabolism of neonatal neutrophils.

Finally, our studies define subpopulations of neonatal neutrophils with respect to basal F-actin content. Subpopulations of adult neutrophils, with respect to histochemical, receptor, and antigenic characteristics have been defined.²¹ In fact, even among adult cells Wallace et al identified fMLP-activated neutrophils that differ in F-actin content and postulated that they are "slower to respond" with fMLP-induced actin polymerization.¹⁷ Our data show that the subpopulations of neonatal neutrophils clearly are not a result of differences in the polymerizability of actin because following fMLP-stimulation, one homogeneous, normally distributed group of cells is observed. As suggested by Wallace et al¹⁷ and discussed above, the differences in basal F-actin content in subgroups of neonatal cells likely reflects a difference in the sensitivity of the cell to activation, and F-actin content simply is a reflection of that activation. The presence of such subpopulations of neonatal neutrophils with respect to cytoskeletal organization invites further investigations of their importance to host defense.

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CONCISE REPORT

The Synthetic RGDS Peptide Inhibits the Binding of Fibrinogen Lacking Intact α Chain Carboxyterminal Sequences to Human Blood Platelets

By Ellinor I.B. Peerschke and Dennis K. Galanakis

The α chain 572-574 Arg-Gly-Asp sequence of fibrinogen appears to play only a minor role in platelet aggregation based on the ability of fibrinogen preparations lacking α chain carboxyterminal segments to support platelet aggregation, but synthetic Arg-Gly-Asp-Ser (RGDS) peptides are capable of inhibiting platelet aggregation and fibrinogen binding. The present study thus examined the ability of RGDS peptides to inhibit platelet interactions with a plasmonic degradation product of fibrinogen (8D-50) that resembles an intermediate fragment X. Gel-filtered, human blood platelets suspended in 0.01 mol/L HEPES-buffered modified

Tyrodé's solution, pH 7.5, were stimulated with 20 μ mol/L adenosine diphosphate and the binding of ^{125} I-labeled 8D-50 or intact fibrinogen (0.01 to 0.6 mg/mL) assessed in the presence of 0 to 117 μ mol/L RGDS. The data revealed that RGDS decreased the apparent affinity of 8D-50 and intact fibrinogen for platelets but did not affect the maximum number of binding sites. RGDS thus appears to be a competitive inhibitor not only of intact fibrinogen ($K_i = 12 \pm 2 \mu$ mol/L) but also of 8D-50 ($K_i = 15 \pm 3 \mu$ mol/L) (mean $\pm$ SD, $n = 3$).

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FIBRINOGEN α chains possess a carboxyterminal amino acid Arg-Gly-Asp (RGD) sequence that is also present on other adhesive glycoproteins including von Willebrand factor (vWF) and fibronectin, all of which appear to bind to the same platelet receptor, the glycoprotein IIb/IIIa complex.^{1,2} Moreover, the synthetic RGDS peptide has been shown to inhibit fibrinogen binding to platelets and platelet aggregation.³ Early studies suggested that the carboxyterminal region of the fibrinogen α chain and/or a segment containing the aminoterminal of the B β chain were involved in platelet fibrinogen interactions since derivatives lacking such segments supported platelet aggregation less well than did more intact fibrinogen.⁴ In apparent contradiction are observations of platelet aggregation supported by fibrinogen preparations with increasing carboxyterminal α chain degradation resulting in α chains of molecular weights ranging from 68,000 to 22,600 as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.⁵ These studies suggested that platelet-fibrinogen interactions were not significantly altered by the loss of carboxyterminal α chain sequences.⁵ Thus, to further characterize the RGDS-induced inhibition of fibrinogen binding, the present study compared the effect of increasing concentrations of RGDS on platelet-fibrinogen interactions in the presence of either intact fibrinogen or an early plasmonic degradation product (8D-50) that lacks the α chain 572-574 carboxyterminal RGD sequence.

MATERIALS AND METHODS

Platelet preparation. Blood was collected from healthy volunteers after obtaining informed consent according to the guidelines of the Declaration of Helsinki. It was anticoagulated with a 0.1 vol of 3.2% sodium citrate. Gel-filtered platelets (GFP) were prepared essentially as described.⁶ They were suspended in 0.01 mol/L hydroxyethyl piperazine-ethane sulfonic acid (HEPES)-buffered modified Tyrodé's solution, pH 7.5.

Fibrinogen preparation and purification. Intact, band I fibrinogen was isolated from fresh-frozen plasma as described.⁵ An intermediate plasmonic degradation product designated 8D-50 was prepared as described⁷ from a 50% clottable plasmonic fibrinogen digest. Both fibrinogens were reduced with 2% β -mercaptoethanol and electrophoresed on 7.0% SDS-polyacrylamide gels.⁸ This disclosed the virtual absence of intact α chains in 8D-50 and a major α chain remnant termed $\alpha/11$. This remnant was previously described to represent the aminoterminal third of the A- α chain and the major and longest molecular weight [MW], 25,000) A- α chain fragment in such preparations.⁹ In addition, a major portion of the β chains had been degraded displaying the $\beta/8$ fragment (MW, 54,700).⁹ Factor XIIIa cross-linked samples also displayed a band slightly cathodal to that of the γ chain corresponding to $\beta/3$ (MW, 51,100). The γ chains of 8D-50 remained intact as assessed by analysis of factor XIIIa cross-linked samples and by the presence of only trace amounts of more anodal non- α chain bands. High-performance liquid chromatography analysis¹⁰ revealed that the A peptide content of 8D-50 was identical to that of intact fibrinogen (2 residues/mol) and its B peptide content was 27% of that of intact fibrinogen, which is consistent with the presence of β chain fragments (see the preceding discussion). Both intact fibrinogen and 8D-50 were iodinated with iodine 125 using chloramine T.⁶

Fibrinogen binding. Fibrinogen binding studies were performed at room temperature using GFP stimulated with 20 μ mol/L adenosine diphosphate (ADP) in the presence of increasing concentrations of 8D-50 or intact fibrinogen and 0 to 117 μ mol/L RGDS. Specific binding was quantified as previously described.⁶ The data were analyzed by double reciprocal plots.¹¹

Platelet aggregation. GFP were aggregated with 20 μ mol/L ADP in the presence of increasing concentrations of intact fibrinogen and 8D-50 (0.01 to 0.20 mg/mL).⁶ Aggregation studies were performed at 37 °C in a dual-channel aggregometer (Chronolog Corp, Havertown, PA) with baselines set at 10% light transmission using GFP and 90% light transmission using a buffer blank.

RESULTS

Figure 1 shows the SDS-polyacrylamide gel electrophoresis profiles of reduced intact fibrinogen and 8D-50. Signifi-

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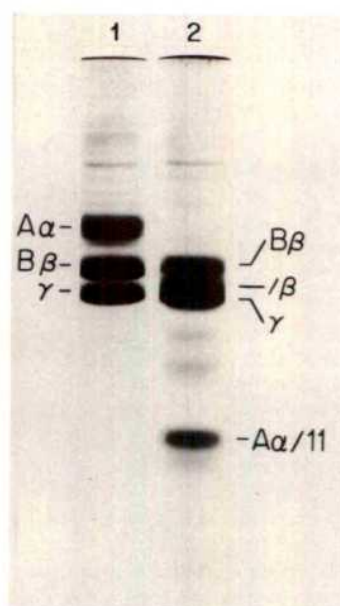


Fig 1. SDS polyacrylamide cylindrical gels (7.0%) depicting the migration of intact fibrinogen (1) and 8D-50 (2) following reduction with 2% mercaptoethanol.

cant α chain degradation is present in the latter with the appearance of a predominant 25,000-MW α chain remnant. Despite this α chain degradation, the ability of 8D-50 to support ADP-induced platelet aggregation was only minimally reduced (Table 1).

The synthetic RGDS peptide inhibited the interaction between ADP-treated platelets and both ^{125} I-labeled intact fibrinogen and 8D-50 with a concentration resulting in 50% inhibition (IC_{50}) of 12.5 μ mol/L (Fig 2). To investigate further the mechanism of this inhibition, the effect of increasing doses (0 to 72 μ mol/L) of RGDS on fibrinogen and 8D-50 binding at various concentrations was quantified. Analysis of the data by double reciprocal plots of (free fibrinogen or 8D-50) $^{-1}$ v (bound fibrinogen of 8D-50) $^{-1}$ indicate that RGDS decreased the apparent affinity of both intact fibrinogen (Fig 3) and 8D-50 (Fig 4) for platelets (X intercept) but did not affect the maximum number of fibrinogen binding sites (Y intercept). Thus, RGDS appears to be a competitive inhibitor of both fibrinogen and 8D-50, even though the latter lacks the α chain carboxyterminal 572-574 RGD sequence. Inhibitor constants calculated from the binding data showed no significant difference between the ability of RGDS to compete with intact fibrinogen (K_i =

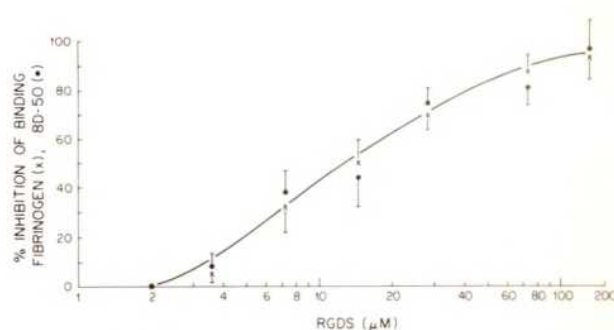


Fig 2. Inhibition of intact fibrinogen and 8D-50 binding (0.6 mg/mL) to ADP-stimulated platelets in the presence of RGDS peptide. Platelets were preincubated (five minutes, 22 °C) with increasing concentrations of RGDS peptide or buffer and inhibition of fibrinogen or 8D-50 binding quantified. (X), intact fibrinogen; (●), 8D-50. Values represent the means $\pm$ SD of five different experiments.

12 $\pm$ 2 μ mol/L) and 8D-50 (K_i = 15 $\pm$ 3 μ mol/L) (mean $\pm$ SD, n = 3), P > .2.

DISCUSSION

The role of fibrinogen α chains in platelet aggregation is controversial. Although the loss of α chain carboxyterminal amino acid sequences has only minor effects on platelet aggregation,⁵ the RGDS tetrapeptide that resembles the carboxyterminal amino acid sequence in position 572-575 of fibrinogen α chains was recently shown to inhibit platelet aggregation and fibrinogen binding.³ Data from the present study suggest that RGDS competitively inhibits the interac-

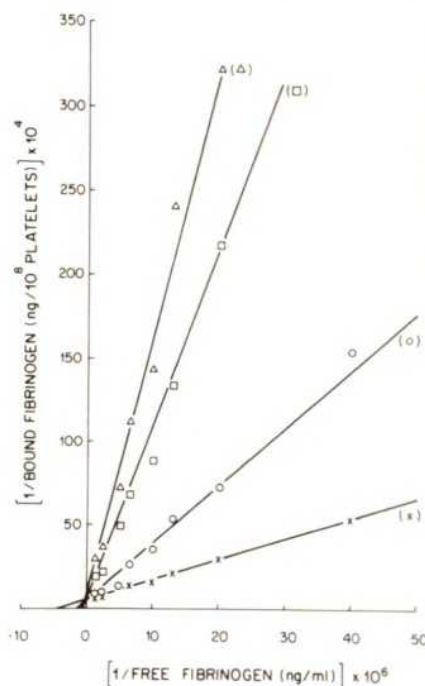


Fig 3. Inhibition of ^{125} I-labeled intact fibrinogen binding to ADP-stimulated platelets by increasing concentrations of RGDS. (X), 0 μ mol/L; (O), 7.2 μ mol/L; (□), 28.8 μ mol/L; (Δ) 72.2 μ mol/L RGDS. Values represent results of a typical experiment.

Table 1. ADP-Induced Platelet Aggregation in the Presence of Intact Fibrinogen or 8D-50

Fibrinogen/ 8D-50 Concentration (μ g/mL)	Platelet Aggregation			
	Rate* (%/min)		Extent* (%)	
	Fibrinogen	8D-50	Fibrinogen	8D-50
25	14 $\pm$ 3	11 $\pm$ 4	16 $\pm$ 5	12 $\pm$ 7
50	20 $\pm$ 6	18 $\pm$ 3	25 $\pm$ 9	20 $\pm$ 8
100	35 $\pm$ 6	30 $\pm$ 8	40 $\pm$ 12	30 $\pm$ 11
200	44 $\pm$ 5	38 $\pm$ 6	60 $\pm$ 15	46 $\pm$ 12

*Mean $\pm$ SD, n = 3.

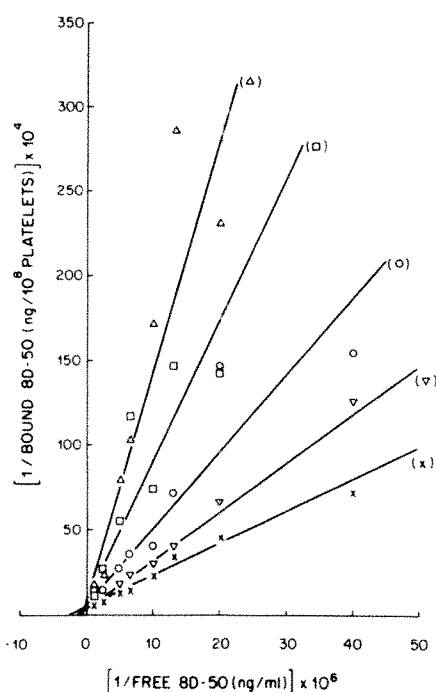


Fig 4. Inhibition of ^{125}I -labeled 8D-50 binding to ADP-stimulated platelets by increasing concentrations of RGDS. (X), 0 $\mu\text{mol/L}$; (▽), 1.44 $\mu\text{mol/L}$; (○), 7.2 $\mu\text{mol/L}$; (□), 28.8 $\mu\text{mol/L}$; (Δ), 72.2 $\mu\text{mol/L}$ RGDS. Values represent results of a typical experiment.

tion between platelets and a fibrinogen preparation (8D-50) that is missing the 572-574 α chain RGD sequence. The inhibitor constant calculated for this interaction was not statistically different from that calculated for RGDS-induced inhibition of intact fibrinogen binding to stimulated platelets.

Although early studies suggested that the carboxyterminal region of the fibrinogen α chain was involved in platelet-fibrinogen interactions, which was based on observations that derivatives lacking such segments supported platelet aggregation as well as more intact fibrinogen,⁴ the extent to which the aggregating activity of fibrinogen was compromised remained controversial.¹² Present data, showing similar platelet aggregation support by 8D-50 and intact fibrinogen and RGDS-induced inhibition of 8D-50 binding, suggest that the 572-574 α chain RGD sequence may play only a minor role in platelet aggregation.

The ability of RGDS to inhibit the binding of fibrinogen lacking intact α chain carboxytermini to ADP-treated platelets indicates that RGDS either blocks fibrinogen interactions with platelets via the α chain RGD sequence at positions 95-97 or via γ chain carboxyterminal regions. In the latter case, the competition between 8D-50 and RGDS for platelet receptors and the recent observation that fibrinogen α and γ chain binding domains may interact with different

regions on the platelet GPIIb/IIIa complex¹³ suggest that these α and γ chain binding regions may be in close proximity. This conclusion is further supported by data indicating that synthetic γ chain peptides inhibit vWF binding to stimulated platelets,¹⁴ although vWF does not possess a γ chain-like amino acid sequence.

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Blast Cell Colony Assay for Umbilical Cord Blood and Adult Bone Marrow Progenitors

By Anne G. Leary and Makio Ogawa

We previously described candidate human blast cell colonies in culture of umbilical cord blood cells. However, their replating efficiencies were low, and we were unable to grow colonies from adult marrow cells. We report here a consistent method of growth and identification of human blast cell colonies that are supported by low serum culture and by delayed addition of medium conditioned by a T lymphoblast cell line, C5MJ. Nonadherent mononuclear cord blood and bone marrow cells were prepared by use of Ficoll-Paque and overnight adherence to plastic. Bone marrow cells were further enriched for progenitors by panning with monoclonal anti-My-10 antibody. Cells were plated in methylcellulose culture containing 2% fetal calf serum and supplemented with bovine serum albumin, lecithin, cholesterol, and transferrin. On day 14 of culture,

concentrated C5MJ-conditioned medium was carefully added to each dish. Blast cell colonies consisting of 18 to 100 cells were detected on days 21 to 28. Forty percent to 75% of the blast cell colonies in individual samples yielded secondary colonies upon replating (positive colonies). The replating efficiency of the positive colonies ranged from 3% to 100%. The largest secondary colony contained 7,800 cells. In addition to single-lineage colonies, multilineage colonies revealing two to five lineage combinations were seen. These results suggest that human primitive progenitors are dormant in cell cycle and that they survive in the absence of colony-stimulating factors. Human blast cell colonies may provide a unique population of progenitors for studies of the early process of human hemopoiesis.

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AMURINE BLAST CELL COLONY assay has proved to be a useful tool for studies of the differentiation and proliferation of primitive hemopoietic progenitors in culture. For example, micromanipulation of individual cells from blast cell colonies provided evidence supporting the stochastic model of commitment of multipotent progenitors to monopotent progenitors.^{1,2} Serial observation of the proliferation of blast cell colonies in culture suggested that primitive stem cells are in G₀ and begin active proliferation randomly.³ Blast cell colonies also provided purified hemopoietic progenitors that were useful for defining the direct actions of colony-stimulating factors (CSF).^{4,5}

Earlier we described candidate human blast cell colonies in culture of umbilical cord blood mononuclear cells in the presence of medium conditioned by phytohemagglutinin-stimulated peripheral blood leukocytes.⁶ However, their incidence and replating efficiencies were low, which limited their usefulness. Furthermore, we were unable to find this type of colony in culture of adult human bone marrow cells. More recently, we observed that murine hemopoietic stem cells that are dormant in cell cycle survive in the absence of interleukin-3 (IL-3) and that they may be preferentially grown in culture in the presence of low concentrations of IL-3.⁷ We have applied this knowledge toward the development of a human blast cell colony assay.

In this paper, we report a consistent method of growth and identification of human blast cell colonies with high replating efficiencies that are supported by low serum culture and by delayed addition of medium conditioned by a human T lymphoblast cell line, C5MJ.

MATERIALS AND METHODS

Cell preparation. Umbilical cord blood and bone marrow samples aspirated from healthy adult volunteers were placed in 16-mL Falcon tissue culture tubes containing 400 units of preservative-free heparin (Invenex Laboratories, Inc, Melrose Park, IL). The donors were advised of the risks of marrow aspiration and gave informed consent. These studies were approved by the Institutional Review Board for Human Research. Mononuclear cells were isolated by the use of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ)

and the nonadherent fraction separated by overnight adherence to plastic. A portion of the samples was further enriched for colony-forming cells by positive indirect immune adherence using the commercial preparation of anti-My-10 monoclonal antibody,⁸ human progenitor cell antibody (Becton Dickinson, Mountain View, CA), and the "panning" technique of Engleman et al.⁹ Details of this procedure have been previously reported.⁸

Primary culture. Nonadherent mononuclear (NAMN) cells and panned My-10+ cells were cultured in 35-mm Lux suspension culture dishes (Miles Laboratories, Inc, Naperville, IL) by a modification of the technique of Iscove et al.¹⁰ One milliliter of culture consisted of 1×10^5 NAMN cord blood cells or 2×10^5 NAMN bone marrow cells or 2×10^4 My-10+ cord blood or bone marrow cells and α -medium (Flow Laboratories, Inc, Rockville, MD) with 25 mmol/L HEPES buffer (Flow), 1.2% of 1,500-centipoise methylcellulose (Fisher Scientific Co, Norcross, GA), 2% fetal calf serum (FCS) (Flow), 1% crystallized and deionized bovine serum albumin (BSA) (Sigma Chemical Co, St Louis), 5×10^{-5} mol/L mercaptoethanol, 300 μ g/mL fully iron-saturated human transferrin (98% pure, Sigma), 160 μ g/mL soybean lecithin (Sigma), 96 μ g/mL cholesterol (Sigma), and 10^{-7} mol/L sodium selenite (Sigma). Dishes were incubated at 37 °C in a humidified atmosphere of 5% CO₂ in air. On day 14 of clonal culture, 100 μ L of concentrated C5MJ-conditioned media (CM) containing 25 mmol/L HEPES buffer was carefully layered over 1 mL of culture.

CM. Media were conditioned by cells of the T lymphoblastoid cell line C5MJ that had been provided by Dr Jun Minowada,

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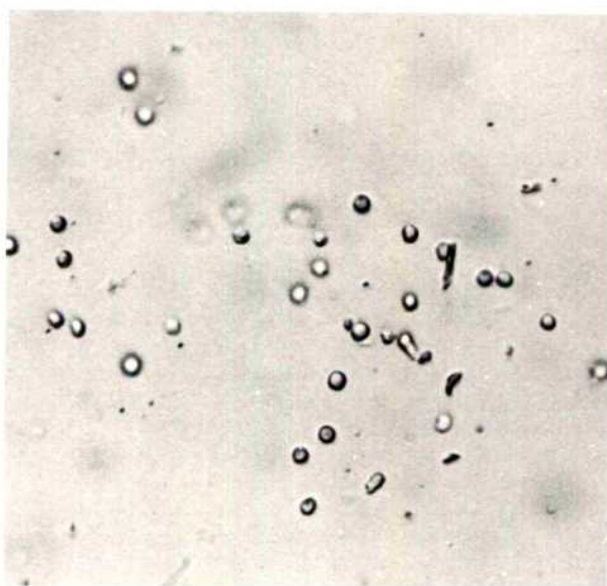


Fig 1. A typical blast cell colony consisting of refractile, round cells that occasionally reveal taillike structures.

Roswell Park Memorial Institute, Buffalo, NY. The cells were originally obtained from patient M.J. with a diagnosis of mycosis fungoides. The human T cell leukemia virus type I-positive cells are the product of the fifth serial passage with cord blood cells. Cells were grown in α -medium containing 5% FCS and the media harvested from roller bottle culture after 1 week. Concentrated media ($30\times$) were obtained by filtration through a 1,000,000-molecular weight membrane followed by concentration through a 10,000-molecular weight membrane using a Pellicon Cassette system. Ten percent of a 1:10 or 1:20 dilution of $30\times$ media was added to each culture.

Replating experiments. On days 21 to 28 of incubation, blast cell colonies were individually lifted from the culture and suspended in 100 μ L of α -medium. The samples were gently pipetted and then added to 0.9 mL of methylcellulose medium containing 30% FCS or a mixture of 15% FCS and 15% fresh human plasma,^{11,12} 2 units of partially purified human urinary erythropoietin (Ep) with a specific activity of 370 U/mg protein (kindly provided by Dr Makoto Kawakita, Kumamoto, Japan), 15% to 30% final concentration of C5MJ CM and 1% fraction V BSA (Sigma). Cultures were incubated at 37 °C and 5% CO₂ in air. When the colonies appeared to have matured (generally after 12 to 16 days of culture), the number of cells of small colonies consisting of <500 cells was estimated in situ, and the entire colony was lifted by use of an Eppendorf micropipette, and smears were prepared using a cytospin

(Shandon Southern Instruments, Sewickley, PA). Larger colonies of >500 cells were picked, made into single-cell suspension, and aliquoted into two half samples. Half of the sample was used for counting in a counting chamber and the other half prepared for cytological examination. Slides were stained with May-Grünwald-Giemsa (Gallard Schlesinger Chemical Manufacturing Corp, Carle Place, NY),¹³ for differential counting.

RESULTS

Colony formation. Blast cell colonies consisting of 18 to 100 cells were detected between days 21 and 28 after cell plating. A picture of a representative blast cell colony is shown in Fig 1. Typically, these colonies are diffuse and consist of refractile, round cells often revealing taillike structures. These cells are also pleomorphic and continue to change their morphology during observation under an inverted microscope. When NAMN cord blood cells were plated in culture, there were macrophage clusters and colonies in addition to the blast cell colonies. However, in cultures of NAMN marrow cells there was macrophage overgrowth that hindered the identification of blast cell colonies. When My-10+ bone marrow cells were cultured, only scattered individual macrophages constituted the background, and this allowed for easy identification of blast cell colonies. Some of the apparent blast cell colonies contained nonrefractile cells that were larger than the typical blasts. These types of colonies eventually developed into macrophage colonies or revealed only a few macrophage clusters upon replating. When these colonies were excluded and only the typical blast cell colonies were replated in cultures containing C5MJ CM and Ep, a varying but high secondary colony formation was observed. A summary of the replating of the individual blast cell colonies from three cord blood samples and three My-10+ bone marrow samples is presented in Table 1. The frequencies of the blast cell colonies that yielded secondary colonies (positive colonies) and their replating efficiencies were variable but high. These features are similar to those of murine blast cell colonies we reported previously.¹⁴ The secondary colonies varied in size, with a maximum of approximately 7,800 cells per colony. The analysis of the types of secondary colonies seen in the replating of blast cell colonies derived from cultures of cord blood (sample 3 of Table 1) and bone marrow (sample 6 of Table 1) is presented in Table 2. In addition to single-lineage neutrophil, macrophage, eosinophil, basophil, and erythro-

Table 1. Summary of Replating of Blast Cell Colonies

Sample	Source of Cells	Size of Blast Cell Colonies	No. Positive Colonies* per Colonies Identified	Replating Efficiency (%) of Positive Colonies
1	CB†	25-75	11/28	8-100
2	CB	16-100	7/11	3-85
3	CB	14-35	10/23	11-90
4	My-10+ BM‡	25-40	8/18	8-41
5	My-10+ BM	18-40	9/12	10-44
6	My-10+ BM	22-33	9/13	10-58

*Positive colonies indicate the blast cell colonies that yielded secondary colonies upon replating.

†Nonadherent cord blood mononuclear cells.

‡My-10+ fraction of nonadherent bone marrow mononuclear cells.

Table 2. Cytological Analysis of Secondary Colonies Seen in Replating of Blast Cell Colonies From a Representative Cord Blood and a Bone Marrow Culture

Lineage Expressed in the Secondary Colonies						No. of Secondary Colonies	Estimated Colony Size (Cells/Colony)
n	m	b	e	E	M		
Cord blood (Sample 3)							
+						1	50
	+					15	40-600
			+			2	150-200
				+		1	1,000
+	+					6	100-400
	+		+			2	250-300
	+			+		1	1,600
		+		+		1	6,000
+	+		+			2	2,000-4,000
+	+			+		1	200
	+	+		+		1	2,000
Total						33	
Bone marrow (sample 6)							
	+					22	30-300
		+				3	50-80
			+			16	50-300
				+		8	200-3,000
+	+					4	70-200
	+		+			1	800
	+			+		1	800
	+		+	+		1	1,000
		+	+	+		1	1,400
+	+			+	+	1	4,000
	+	+	+	+		2	600-3,000
+	+	+	+	+		1	1,400
Total						70	

The secondary colonies were derived from replating all blast cell colonies seen in the culture of samples 3 and 6 (see Table 1).

Abbreviations for lineages proposed at a UCLA symposium: n, neutrophils; m, monocyte-macrophages; b, basophils; e, eosinophils; E, erythrocytes; M, megakaryocytes.¹⁵

cyte colonies, multilineage colonies of two- to five-lineage combinations were seen.

DISCUSSION

We previously reported that culture of marrow cells from normal mice in the presence of low FCS combined with the delayed addition of IL-3 favors blast cell colony formation.⁷

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In the current report, we describe a similar cell culture technique that favors human blast cell colony formation from umbilical cord blood and adult bone marrow cells. Since human IL-3 was not yet available, we chose to use CM prepared from the human lymphoblast cell line C5MJ. Our observation that the addition of CM to low serum cultures 2 weeks after cell plating supported blast cell colony formation suggests that human multipotent progenitors are also dormant in cell cycle and do not require CSFs until they begin active cell proliferation. Our data also suggest that the more mature progenitors that are in active cell cycle appear to require the presence of CSFs for their continued proliferation and survival. According to a rough estimate, there was one blast cell colony-forming cell per 5×10^4 NAMN cord blood cells. There were approximately one to two blast cell colony-forming units per 1×10^4 My-10+ adult bone marrow cells. Since panning with anti-My-10 antibodies usually yields 1% of the original NAMN cells, the incidence of blast cell colony-forming cells in adult bone marrow cells was estimated to be one to two per 10^6 NAMN bone marrow cells. Blast cell colonies were detected only in cultures of My-10+ populations of adult human bone marrow cells but not NAMN marrow cells. This is a technical problem created by a high background macrophage colony growth from impure cell populations.

Although the cells that form blast cell colonies are likely to be in G_0 and begin active proliferation randomly, their exact relationship with human pluripotent stem cells has not been clarified. A consistent human blast cell colony assay is likely to contribute to our better understanding of the properties of human hemopoietic stem cells. Recently, many human CSFs have been purified and/or molecularly cloned. Furthermore, complex interactions among various types of growth factors including CSFs are being uncovered. Human blast cells will offer a unique purified population of hemopoietic progenitors for defining the direct roles of human CSFs. In addition, a consistent assay for the most primitive human progenitors to be identified in culture may be useful for elucidation of the pathophysiological mechanisms of certain hemopoietic disorders.

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Cell Surface Phenotyping of Megakaryoblasts

By Tadashi Koike, Sadao Aoki, Souichi Maruyama, Miwako Narita, Toshie Ishizuka, Hirofumi Imanaka, Toyohiko Adachi, Hiroo Maeda, and Akira Shibata

Surface phenotypic characterization of megakaryoblasts, identified by platelet peroxidase activity, was investigated in four patients who showed increased proliferation of megakaryoblasts: one patient with typical features of acute leukemia, one presenting with acute myelofibrosis, and two with Down's syndrome in whom blasts disappeared spontaneously (transient abnormal myelopoiesis, TAM). MY10 and/or MY9 antigens were expressed on the surface of some megakaryoblasts, but MY7, and MY4, antigens specific to granulocytic or monocytic cells, were

not. Some megakaryoblasts were positive for only anti-HLA-DR antibodies. It was speculated that, during the differentiation of the megakaryocytic lineage, MY9 antigen appears transiently on the surface of megakaryoblasts that have lost HLA-DR antigens and have gained the glycoprotein IIb/IIIa antigen. This study also demonstrated that the proliferating blasts in some patients with TAM were mainly megakaryoblasts and suggested that the target cells in TAM are CFU-GEMM.

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THE USE OF SPECIFIC monoclonal antibodies has provided important insights into leukocyte differentiation and cellular origin in leukemia.¹ These antibodies were found to be very useful in investigating the stages of differentiation in leukemic blasts. However, little is known about the cell surface phenotypes of megakaryoblasts because of the rarity of megakaryoblastic leukemia.

Platelet peroxidase (PPO), a specific enzymatic marker of megakaryocytic cell lineage, is detected in early megakaryocytes and appears earlier than platelet-specific glycoproteins (GP), such as the GP IIb/IIIa complex or GP Ib, or storage proteins in platelets, such as von Willebrand factor antigens.²⁻⁵ In this study, cell surface phenotypes were examined in patients with megakaryoblastic leukemia in whom the proportions of blast cells were more than 45% and PPO activity was detected in more than 65% of the blasts.

MATERIALS AND METHODS

Patients. Clinical data on all patients are shown in Table 1. Patient 1 was an infant without Down's syndrome. Aspiration of bone marrow was easy, and the presence of myelofibrosis was unlikely. Patient 2 presented with acute myelofibrosis. Circulating blasts increased gradually. Patients 3 and 4 had Down's syndrome with a spontaneous disappearance of proliferating blasts. Regarding the aspiration and biopsy of bone marrow, all patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent.

Blood smears and cytochemical studies. Blood smears were stained with May-Grünwald-Giemsa. Cytochemical reactions for myeloperoxidase (MPO), Sudan black B, α -naphthyl acetate esterase, α -naphthyl butyrate esterase, acid phosphatase, and periodic acid-Schiff (PAS) were performed by conventional methods.⁶

Electron microscopic studies. PPO was detected by the method of Anderson et al⁷ or Breton-Gorius et al² with a slight modification as described elsewhere.⁵ More than 200 blasts were examined, and the blasts positive only in perinuclear space or endoplasmic reticulum were regarded as PPO-positive. Positive blasts in granules or Golgi cisternae were regarded as MPO-positive.

Cell surface phenotyping. For the detection of surface antigens, samples were analyzed using a Spectrum III (Ortho Diagnostics, Westwood MA). A population of blast cells was clearly distinguished from the lymphocytes, monocytes, and granulocytes on the basis of light-scattering properties. Monoclonal antibodies used in this study were as follows: OKT3 (CD3), OKT11 (CD2), OKT4 (CD4), and OKT8 (CD8) purchased from Ortho Pharmaceutical Corp, Raritan, NJ; J5 (CD10), B4 (CD19), B1 (CD20), MY7

(CDw13), MY4 (CDw14), MY9, and MY10 purchased from Coulter Immunology, Hialeah, FL; HLA-DR and LeuM3 purchased from Becton Dickinson, Mountain View, CA; and TP80 (IgG1), which detects GP IIb/IIIa antigen,^{8,9} and another monoclonal anti-GPIIb/IIIa antibody, PLT-2, purchased from Cappel Laboratories, Cochranville, PA. The positivity for the monoclonal anti-GPIIb/IIIa antibodies was also examined by an indirect immunalkaline phosphatase technique⁵ or by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method.¹⁰ Fc binding of these antibodies to the stained cells was excluded by the absence of reactivity with monoclonal antibodies directed to unrelated antigens of the same immunoglobulin subclasses and by the findings that the saturation of Fc receptors by preincubation with normal rabbit serum had no effect on the APAAP staining results obtained using monoclonal antibodies to GP IIb/IIIa. The results of indirect immunalkaline phosphatase staining conjugated F(ab')₂ immunoglobulin were almost the same as those of APAAP staining. Terminal deoxynucleotidyl transferase (TdT) activity was detected by immunofluorescence using a Bethesda kit (Bethesda Research Laboratories Rockville, MD).

RESULTS

Light microscopic cytochemistry. Almost all of the patients' blasts were negative for peroxidase, Sudan black B, and α -naphthyl butyrate staining but were positive for acid phosphatase. Blasts in patient 1 stained strongly for α -naphthyl acetate, whereas blasts in the other patients stained weakly for this enzyme. Some blasts from all patients were positive for PAS.

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Table 1. Clinical and Cytological Findings of Four Patients

Markers	Patients			
	1	2	3	4
Age	1 yr	15 yr	<1 mo	<1 mo
WBC (10^9)		4.7	118.0	172.0
Blasts (%)	95 (BM)	67	88	46
PPO (%)	86	82	68	80
MPO	1	0	2	4
PO (-)	13	18	30	16
TP80 (%)	83	1	42	16
PLT-2	NT	1	NT	14
MY10	86	NT	NT	71
MY9	93	1	48	33
MY7	3	0	4	3
MY4 (LeuM3)	3	(4)	4	2
HLA-DR	8	63	38	24
J5	2	3	1	2
TdT	—	—	—	—

Percentages of cells positive for ultrastructural cytochemistry and immune markers refer to the percentage of blasts as assessed by their characteristic morphology and by size gating (see Materials and Methods).

Abbreviations: BM, bone marrow; PO(-), blasts negative for peroxidase activity; TP80 and PLT-2, antiplatelet GP IIb/IIIa antibodies; NT, not tested.

Electron microscopic cytochemistry. In all patients more than 65% of the blasts were positive for PPO in perinuclear space and endoplasmic reticulum (Table 1, Fig 1). Less than 5% of the blasts were positive for peroxidase in granules and Golgi apparatus.

Cell surface phenotyping. The immunologic markers of the blasts are shown in Table 1. Most of the blasts from patient 1 reacted to TP80 (Fig 2), whereas most of the blasts from patient 2 were negative for this antibody. The proportions of cells positive for TP80 were similar to those of PPO-positive cells in patients 1 and 3, whereas in patients 2 and 4, the proportions of TP80-positive cells were rather low compared with those of PPO-positive blasts. In all patients, the blasts were negative for lymphoid markers such as B2, B4, OKT3, OKT11, OKT4, OKT8, J5, and TdT. In contrast, a considerable proportion of the blasts were positive for

MY10 and MY9 in patients 1, 3, and 4, but their blasts were rarely positive for MY7 and MY4. In patient 2, the blasts were negative for MY9 and TP80 but positive for HLA-DR, whereas in patient 1, they were positive for both MY9 and TP80 but negative for HLA-DR.

DISCUSSION

We encountered four patients in whom the proliferating blasts were recognized as megakaryoblasts, although the patients' clinical features varied. For identification of megakaryoblasts, detection of PPO activity is necessary when the proportion of blasts positive for antiplatelet antibodies is small because PPO activity appears earlier than platelet-specific glycoproteins such as GP IIb/IIIa antigens during the process of megakaryocyte maturation.³⁻⁵ In this study, we examined whether other markers, especially myeloid markers, are expressed on megakaryoblasts with PPO activity. A considerable proportion of blasts was positive for MY10 and/or MY9 in patients 1, 3, and 4, whose blasts were rarely positive for MY7 or MY4. It is noteworthy that most of the blasts in patient 1 were positive for MY10 and MY9 in addition to high proportions of cells positive for anti-GPIIb/IIIa and PPO activity. MY10 antigen is a cell surface protein with an apparent molecular weight of 115 kilodaltons and was produced after immunization with KG-1a human leukemia cells.¹¹ Cell sorting and panning experiments demonstrated that CFU-GM and BFU-E were MY10-positive. The MY9 monoclonal antibody was produced by Griffin et al¹² following immunization with the cells of a patient in the blast crisis of chronic granulocytic leukemia. MY9 antigen is expressed on CFU-GM, myeloblasts, promyelocytes, myelocytes, and monocytes. Approximately 50% of BFU-E and some of CFU-GEMM are also MY9-positive, although more mature erythrocytic cells are negative for this antigen.^{12,13} The present results suggest that megakaryoblasts, which are megakaryocyte precursors, express MY10 and MY9, although mature megakaryocytes and platelets are negative for these antibodies. The blasts of our patients were rarely positive for MY7 and MY4, which are markers of more myeloid development. MY7 antigen is specific to granulocytic and monocytic cells.¹⁴ MY4 antigen is expressed

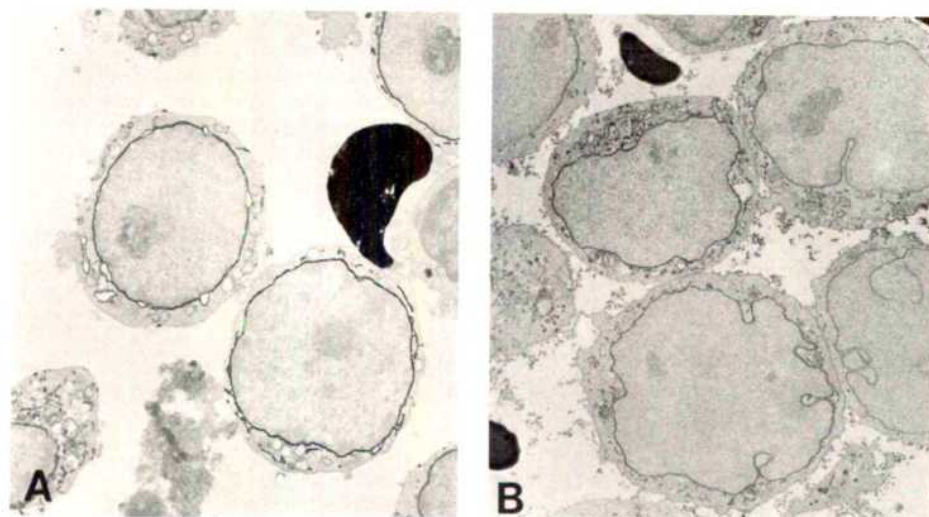


Fig 1. Electron micrographs of megakaryoblasts from the blood of patients 2 (left) and 4 (right). Reaction products for platelet peroxidase are present in the nuclear envelope and endoplasmic reticulum in megakaryoblasts. Unstained sections; original magnifications $\times 6000$ (A) and $\times 5000$ (B).

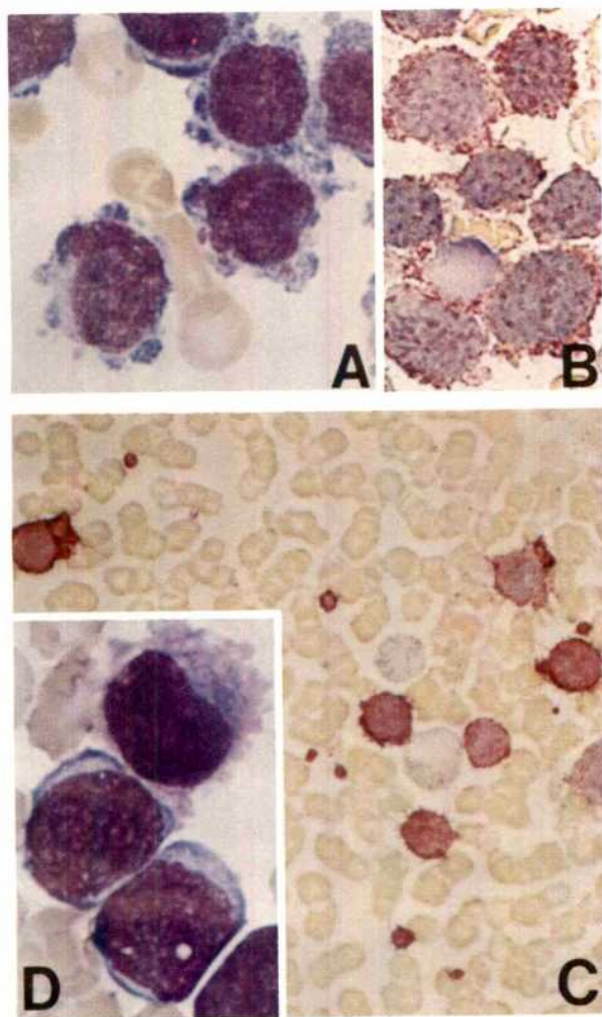


Fig 2. A marrow smear from patient 1 showing replacement of marrow by leukemic blasts (May-Grünwald-Giemsa stain). Most of the blasts have prominent cytoplasmic blebs. (B) A marrow smear from patient 1 showing positive reddish granular staining of GPIIb/IIIa antigen on the surface of all the blasts (APAAP stain using TP80 and fast red TR salt as the color reagent). (C) A blood smear from patient 3 showing reaction for TP80 (APAAP stain). Most of the blasts are strongly positive. (D) Blasts from patient 3 (May-Grünwald-Giemsa stain). Two blasts have a smooth margin without blebs, and one has protrusions.

strongly on monocytic cells and weakly on granulocytic cells.¹⁴

There was heterogeneity of surface marker expression (GPIIb/IIIa, MY10/MY9, and HLA-DR) among patients. This heterogeneity may be related to the level of maturation. Figure 3 illustrates the possible order of appearance of markers in the maturation of the megakaryocytic cell lineage based on the present data. PPO activity was detected in immature megakaryoblasts that retain HLA-DR antigens and do not express MY9 antigen. MY9 antigen may appear on megakaryoblasts that have lost HLA-DR antigens and have expressed GPIIb/IIIa antigens.

The proposed model is based on the data from leukemic megakaryocytic precursors. Therefore, the relationship between the level of megakaryocytic maturation and surface

	Megakaryoblast	Megakaryocyte
HLA-DR	=====	
MY9/MY10	=====	
GPIIb/IIIa	=====	
PPO		=====

Fig 3. The possible order of appearance of markers in the maturation of megakaryoblasts.

marker expression should be confirmed in megakaryocyte colony-forming assays using normal bone marrow cells treated with monoclonal antibodies and complements.

A self-limited blastic proliferation, indistinguishable from acute leukemia except for its disappearance without therapy, has been noted in infants with Down's syndrome.¹⁵ This phenomenon is designated as transient abnormal myelopoiesis (TAM).¹⁶ In addition to previous reports that proliferating blasts in TAM were usually myeloblasts, the results found in patients 3 and 4, ie, mainly the megakaryocytic nature of proliferating blasts, suggest that the target cells in TAM are CFU-GEMM.

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CONCISE REPORT

Molecular Analysis of Interferon-Induced Suppression of Philadelphia Chromosome in Patients With Chronic Myeloid Leukemia

By G. Yoffe, M. Blick, H. Kantarjian, G. Spitzer, J. Gutterman, and M. Talpaz

Treatment with recombinant human interferon alpha-A (Roferon-A) is associated with stable suppression of the population of cells that display the Philadelphia (Ph¹) chromosome in some patients with chronic myelogenous leukemia (CML) as defined by cytogenetic analysis. Southern blot analyses employing a 3' breakpoint cluster region (*bcr*) probe (Pr-1) were performed to confirm a complete suppression of the Ph¹⁺ chromosome-positive clone of cells at the DNA level. The complete disappearance of rearranged

restriction fragments of the *bcr* gene, which were a characteristic of the disease prior to Roferon-A therapy, was accompanied by the restoration of normal bone marrow and achievement of durable ongoing complete remission for 9 and 6 months, respectively, in two patients with Philadelphia-positive (Ph¹⁺) CML. Molecular analysis is a valuable probe for monitoring the clinical course of disease in patients with Ph¹⁺ CML.

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THE ANTIPROLIFERATIVE activity of human alpha interferon has been demonstrated in a number of human tumors *in vivo*¹ as well as in normal myeloid and myeloid leukemic progenitor cells *in vitro*.^{2,3} Recombinant human interferon alpha-A (Roferon-A) has recently been shown to induce hematologic remission in most of the patients with chronic myelogenous leukemia (CML) in the early benign phase.⁴ Therapy with Roferon-A resulted in durable Philadelphia (Ph¹) chromosome suppression (cytoconversion) in some of these patients. Other studies have also demonstrated a complete, albeit transient, suppression of the Ph¹⁺ clone of cells following intensive chemotherapy.⁵

Because more than 90% of the cases of CML display the Ph¹ chromosome, which is the result of a 9:22 translocation, this constitutes a hallmark of the disease.⁶ The *c-abl* oncogene, located on chromosome 9, and the breakpoint cluster region (*bcr*) gene, located on chromosome 22, are juxtaposed in the Ph¹⁺ CML,^{7,8} thus producing a *bcr/abl* chimeric mRNA transcript^{9,10} and a chimeric fusion protein.^{11,12} Upon the introduction of molecular probes capable of identifying this alteration,^{8,13} molecular analysis along with cytogenetic analysis⁶ can be used for monitoring therapies with interferon, intensive chemotherapy, and other treatment modalities throughout the course of the disease in patients with CML.

In this study, suppression of the Ph¹ chromosome clone of cells in two patients with Ph¹⁺ CML following treatment with Roferon-A was accompanied by the loss of an aberrant restriction fragment of the *bcr* gene, which was characteristic of the disease prior to Roferon-A therapy. This provides evidence at the DNA level for suppression of the Ph¹⁺ clone of cells in two patients with CML treated with Roferon-A.

MATERIALS AND METHODS

Case Reports

Patient 1. The diagnosis of Ph¹⁺ CML was made in this 65-year-old Latin American female in September 1984. She remained untreated until December 1984 when daily intramuscular injections of 5×10^6 U/m² of Roferon-A were started. Complete hematologic remission was observed after 8 weeks of treatment, which was manifested by a decrease in the WBC count from $116 \times 10^3/\mu\text{L}$ to $3.4 \times 10^3/\mu\text{L}$ and a return of an enlarged spleen to normal size. One hundred percent of the cells in the blood and bone marrow were initially Ph¹⁺. A bone marrow cytogenetic study following 3 months of therapy demonstrated a decrease of the Ph¹⁺ cells to 30%. A repeat bone marrow study following 6 months of treatment

demonstrated complete suppression of the Ph¹⁺ clone of cells, a finding that persisted in two additional studies at 9 and 12 months following the initiation of therapy. Thus the Ph¹⁺ cells remained continuously suppressed for 9 months.

Patient 2. The diagnosis of Ph¹⁺ CML was made in this 32-year-old white female in June 1984. She was treated initially with hydroxyurea for 2 months, and in October 1984, daily intramuscular injections of 5×10^6 U/m² of Roferon-A were started. Prior to treatment with Roferon-A, the patient's peripheral WBC was $18.8 \times 10^3/\mu\text{L}$, and her platelet count was $566 \times 10^3/\mu\text{L}$. The bone marrow cellularity was 100%, and 100% of the cells were Ph¹⁺. The hematologic parameters of her disease improved gradually, and hematologic remission was achieved 7 weeks after the Roferon-A therapy was started. At that time, her WBC decreased to $3.4 \times 10^3/\mu\text{L}$, and her platelets decreased to $125 \times 10^3/\mu\text{L}$. Subsequently, after 14 weeks of therapy, the treatment dose of Roferon-A was reduced to 2.5×10^6 U/m² when the bone marrow cellularity decreased to 40% and cytogenetic analysis demonstrated a decrease of the Ph¹⁺ cells to 70%. The Ph¹⁺ clone of cells decreased further to 30% at 6 months and to 10% at 9 months of therapy. Complete suppression of the Ph¹⁺ clone of cells occurred after 12 months of therapy and has been sustained after 15 and 18 months after therapy was started.

DNA Isolation and Southern Blotting

Blood and bone marrow samples were obtained from patients after they had been fully advised of the procedure and attendant risks in

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accordance with institutional guidelines and had given informed consent. High-molecular weight DNA was extracted from peripheral blood leukocytes and bone marrow cells by methods described elsewhere.¹⁴ The digested DNA was electrophoresed in a 0.8% agarose gel in a buffer containing 0.4 mol/L Tris, 20 mmol/L EDTA, and 100 mmol/L Na acetate, denatured, neutralized, and transferred to nitrocellulose filters by the Southern blot method.¹⁵ Prehybridization and hybridization were carried out as described previously.¹⁵ A *bcr* probe (Pr-1) purchased from Oncogene Sciences (Mineola, NY) and corresponding to the breakpoint cluster region on chromosome 22⁸ was labeled to a specific activity of 1 to 3×10^8 cpm/ μ g of DNA by nick translation. Following hybridization, the filters were washed under stringent conditions, air-dried, and exposed to x-ray film for one to five days at -70°C .

RESULTS

High-molecular weight DNA was obtained from the peripheral blood of both patients before treatment with Roferon-A and from the peripheral blood of a normal donor. The DNAs were digested by *Bam*HI and *Bgl*II restriction endonucleases and analyzed by Southern blot using a 3' *bcr* probe (Pr-1). Analysis of the DNA from patient 1 demonstrated abnormal 11-kb *Bam*HI and 9-kb *Bgl*II hybridizing fragments in addition to the normal 3.3-kb *Bam*HI and 5-kb *Bgl*II *bcr* fragments (Fig 1, panel 1). Similarly, patient 2 disclosed abnormal 7.6-kb *Bam*HI and 9-kb *Bgl*II hybridizing fragments in addition to the normal *bcr* restriction fragments (Fig 1, panel 2). Repeat analysis of the DNA from both patients' peripheral blood was carried out on samples obtained 12 months after Roferon-A therapy was started. This analysis disclosed that the rearranged hybridizing fragments were not detected by Southern blot using the same *bcr* probe (Fig 2, panel 2). Twelve months after Roferon-A therapy was started, bone marrow DNA was also obtained from patient 1, and a similar Southern blot analysis disclosed no *bcr* rearrangement (Fig 2, panel 1). To determine the sensitivity of our Southern blot assay, a mixing experiment was performed (Fig 3). Using DNA from a normal donor and DNA from a CML patient with an additional abnormal 3-kb *Bgl*II restriction fragment, we found that as little as 5%

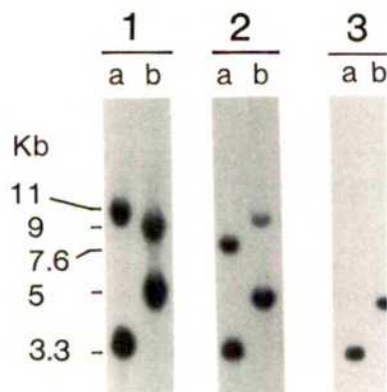


Fig 1. Southern blot analysis demonstrating the normal 3.3-kb *Bam*HI (lane 3a) and 5-kb *Bgl*II (lane 3b) hybridizing fragments in DNA obtained from a normal volunteer and both the normal and rearranged hybridizing fragments in DNA obtained from the leukemic cells of patient 1 (panel 1) and patient 2 (panel 2).

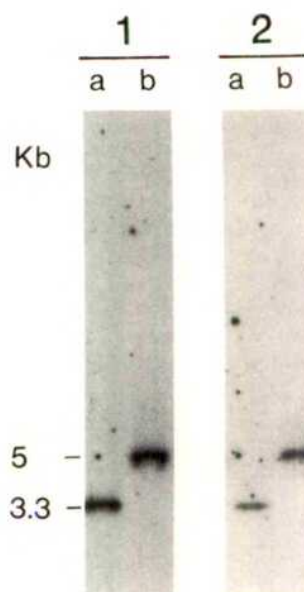


Fig 2. Southern blot analysis demonstrating the normal 3.3-kb *Bam*HI (lane a) and 5.0-kb *Bgl*II (lane b) and the loss of the rearranged *bcr* fragments in the cells obtained from the bone marrow of patient 1 (panel 1) and peripheral blood of patient 2 (panel 2) after both patients had received 12 months of Roferon-A therapy.

contamination of DNA from the Ph^{1+} cells was clearly detectable as a faint band after a long exposure (120 hours). Therefore, in this assay, the level of detection was approximately 5% by Southern blot analysis.

DISCUSSION

We have demonstrated the disappearance of the Ph^{1+} clone of cells in two patients with CML following treatment with Roferon-A. In addition to cytoconversion of the Ph^1 chromosome in all analyzed metaphases, a rearranged *bcr* DNA fragment characteristic of the patients' disease prior to Roferon-A therapy completely disappeared at a time coincidental with hematologic remission.

Previous studies have demonstrated the restoration of nonclonal hematopoiesis in patients with CML following intensive chemotherapy.¹⁶ Similarly, we have reported partial and complete suppression of the Ph^{1+} clone after alpha interferon therapy.^{4,17} In this study, we confirm that the clone



Fig 3. Southern blot analysis of a mixing experiment demonstrating the sensitivity of our assay. DNA (10 μ g) from a normal volunteer was analyzed using the *Bgl*II restriction endonuclease and the *bcr* probe (lane 1). DNA (10 μ g) from a patient with CML was analyzed in a similar fashion (lane 9) and then serially diluted so that 50% of the DNA in lane 8 is from the patient with CML, 25% in lane 7, 10% in lane 6, 5% in lane 5, 2.5% in lane 4, 1% in lane 3, and 0.5% in lane 2.

of cells bearing a DNA rearrangement associated with the formation of the Ph¹ chromosome¹⁸ in CML disappeared from the patients' blood and bone marrow. We have further demonstrated that as little as 5% Ph¹⁺ cells in any given sample can be detected by using Southern blot analysis. The possibility exists that the Ph¹⁺ clone was still present in less than 5% of the bone marrow cells.

Although the Ph¹ chromosome and rearranged *bcr* gene are associated with more than 90% of the cases of CML,^{8,19} it is still unknown whether suppression of the clone bearing the rearranged gene and the Ph¹ chromosome restores the bone marrow to a normal polyclonal pattern. In this instance, a second clonal marker such as the glucose-6-phosphate dehydrogenase isoenzyme pattern²⁰ or the restriction fragment length polymorphism of the hypoxanthine-guanine phosphoribosyl-transferase gene²¹ may help determine whether the

cells that remain when patients are in remission represent the same abnormal clone and simply lack the Ph¹ chromosome and the *bcr* rearrangement. However, since the clinical and hematologic features of CML disappeared completely in these two patients, it is unlikely that the hematopoietic cells of these patients in remission still consist of a monoclonal population.

Demonstration of the suppression of the Ph¹⁺ clone by cytogenetic analysis may reflect only the events in a specific cell population that happens to enter mitosis. Molecular analysis, however, circumvents this problem and provides information on the total cell population (dividing and nondividing cells as well). Therefore, the demonstration of complete suppression of an existing gene rearrangement supports the notion that these patients with CML were in complete remission at the molecular level.

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Differences in Coagulant and Fibrinolytic Activities of Cultured Human Endothelial Cells Derived From Omental Tissue Microvessels and Umbilical Veins

By Wolfgang Speiser, Elisabeth Anders, Klaus T. Preissner, Oswald Wagner, and Gert Müller-Berghaus

Large vessel and microvascular endothelial cells were compared in their capacity to synthesize and secrete coagulant and fibrinolytic factors. Human omental tissue microvascular endothelial cells (HOTMEC) and human umbilical vein endothelial cells (HUVEC) were isolated, grown to confluency under identical conditions, and studied in primary cultures. After an incubation period of 12 hours in serum-free medium, the conditioned medium of confluent HOTMEC contained 100-fold higher levels of tissue plasminogen activator (tPA) antigen than that of HUVEC. The conditioned media as well as the lysates of both cell types did not contain any free tPA activity, but the free plasminogen activator inhibitor capacity was found

intracellularly as well as extracellularly. Although von Willebrand factor was detected in both cell types by immunofluorescence, measurable amounts were only found in HUVEC using an enzyme-linked immunosorbent assay. The kinetics of protein C activation by thrombin on the surface of once-passaged cells were identical for HOTMEC and HUVEC. The present study indicates that cultivated HOTMEC produce larger quantities of tPA than HUVEC do, possess smaller amounts of von Willebrand factor than HUVEC do, and express thrombomodulin for protein C activation as effectively as HUVEC.

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ENDOTHELIAL CELLS are involved in the regulation of coagulation and fibrinolysis. These cells are known to synthesize and release components of the coagulation system such as tissue factor,¹ von Willebrand factor (vWF),² and factor V³ as well as components of the fibrinolytic system such as tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI).⁴ Quiescent, nonstimulated endothelial cells release an excess of fast-acting PAI over tPA, and therefore tPA antigen but almost no free tPA activity is found in the blood^{5,6} as well as in endothelial cell-conditioned medium.⁴

The expression of thrombomodulin on the endothelial cell surface facilitates the activation of protein C by thrombin, which binds to thrombomodulin, thereby losing its fibrin-generating potency. Activated protein C degrades activated factor V and factor VIII:C.⁷ Furthermore, activated protein C may enhance fibrinolysis by limiting PAI activity.⁸

In the present study some significant coagulant and fibrinolytic activities of human umbilical vein endothelial cells (HUVEC) were compared with those of human omental

tissue microvascular endothelial cells (HOTMEC) by using immunologic as well as functional assay systems. It was found that HOTMEC generate larger amounts of tPA and PAI than HUVEC, whereas HUVEC possess more vWF. Protein C seems to be equally well activated by thrombin on the surface of HOTMEC as on that of HUVEC.

MATERIALS AND METHODS

Cultivation of endothelial cells. HOTMEC were isolated as described previously.^{9,10} Briefly, 10 to 15 g human omental tissue was obtained from patients undergoing cholecystectomy. All patients were advised of procedures and attendant risks by the surgeon, in accordance with the institutional guidelines, and gave informed consent. The tissue was dissected into small pieces and incubated in phosphate-buffered saline (PBS) containing 2 mg/mL collagenase (GIBCO, Karlsruhe, FRG) for 15 minutes at 37 °C. After the removal of undissociated tissue fragments by a nylon screen (diameter Ø, 400 µm), capillary fragments were separated from contaminating cells by a further filtration step (nylon screen; Ø, 30 µm) and then by velocity sedimentation at unit gravity (PBS containing 5% bovine serum albumin [wt/vol]). HUVEC were isolated according to Jaffe et al.¹¹ The cells obtained by these procedures were identified as endothelial cells by their typical cobblestonelike growth pattern, by positive immunofluorescence staining for vWF and by immunoreactivity with a monoclonal antibody directed against endothelial cells.¹⁰ Both cell types were seeded on Petri dishes (35 × 10 mm; Becton Dickinson, Heidelberg, FRG) precoated with fibronectin (Behringwerke, Marburg, FRG) and grown to confluency in Waymouth's MB 752/1 culture medium supplemented with 20% (vol/vol) fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine (all culture medium reagents were from GIBCO), and 100 µg/mL endothelial cell growth supplement (Sigma Chemical Co, Munich).

Incubation of endothelial cells in serum-free medium. Four to six days after seeding, two dishes with confluent monolayers of endothelial cells derived from a single umbilical cord or from omental tissue of a single donor were incubated in serum-free medium (SFM) containing 1% (wt/vol) human serum albumin (Sigma) and 2 mmol/L D(+)-glucose (Merck, Darmstadt, FRG). Aliquots of the supernatants were taken at six and 12 hours, centrifuged at 180 g, and stored at -30 °C up to 8 weeks until assessment. At the end of the 12-hour incubation, the cells were washed with SFM and detached from the dishes by incubation with

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1 mL 150 mmol/L NaCl, 0.05% (wt/vol) trypsin, and 1 mmol/L EDTA for two minutes at 37 °C. Thereafter, trypsin was inhibited by adding 1 mL SFM containing 6 mg/mL soya bean trypsin inhibitor (GIBCO). For the assessment of tPA antigen, PAI capacity, and vWF antigen in the cells, an aliquot was removed for determination of the cell count, and the remaining suspension was centrifuged at 180 g for five minutes (the supernatants contained $5.2\% \pm 2.1\%$ [mean $\pm$ SD, $n = 8$] of the amount of tPA antigen (HOTMEC) and $30.1\% \pm 9.2\%$ of the amount of vWF [HUVEC] measured in cell lysates). The cells were then resuspended in 1 mL 0.1 mol/L Tris-HCl, 0.1% (wt/vol) Tween 80, 0.01% (wt/vol) sodium azide, pH 7.42, and sonicated by a cell disruptor (Branson, Soest, The Netherlands) for one minute at 4 °C. After centrifugation at 180 g for five minutes, the supernatants were stored at -30 °C up to 8 weeks until assessment.

tPA and PAI. tPA antigen was measured by an enzyme-linked immunosorbent assay (ELISA) as described earlier.⁵ Reverse fibrin autography and determination of PAI activity were performed as described elsewhere.⁶

vWF. vWF antigen was measured by an ELISA (Boehringer Mannheim, Mannheim, FRG). One unit of vWF was defined as the amount of antigen contained in 1 mL of pool plasma. Immunofluorescence was performed as described elsewhere.¹⁰

Activation of protein C on the endothelial cell surface. Confluent monolayers of pooled and once-passaged cells isolated from several umbilical cords or from omental tissue of several donors were used for the experiments, which were performed as described elsewhere.¹⁰

RESULTS

tPA and PAI. Determinations were performed on individual primary cultures of endothelial cells isolated from omental tissue (HOTMEC) of eight donors and from eight different umbilical cords (HUVEC). In the course of 12-hour incubation in SFM, tPA antigen levels increased in the endothelial cell-conditioned medium (ECCM) of HOTMEC as well as in that of HUVEC (Fig 1). tPA levels in ECCM of HOTMEC (45.1 ± 11.9 ng/ 10^6 cells; mean $\pm$ SD; $n = 8$) were about 100-fold higher than in ECCM of HUVEC (0.41 ± 0.15 ng/ 10^6 cells) at the end of the incubation period. When the total amount of tPA antigen (ECCM plus cell lysates) measured in HOTMEC (62.5 ng/ 10^6 cells) was compared with that of HUVEC (0.66 ng/ 10^6 cells), a 100-fold excess of HOTMEC over HUVEC was calculated. Free tPA activity was not detectable in ECCM and cell lysates of HOTMEC or of HUVEC, but the free PAI capacity (PAI-cap) was found to increase in the course of incubation (Fig 2). After an incubation time of 12 hours, the PAI-cap amounted to 91.5 ± 11.0 U/ 10^6 cells in the lysates and 56.1 ± 9.8 U/ 10^6 cells in ECCM of HOTMEC and to 15.9 ± 7.9 U/ 10^6 cells in the lysates and 21.4 ± 17.1 U/ 10^6 cells in ECCM of HUVEC. The molecular weights (mol wt) determined by reverse fibrin autography of PAI in ECCM of HUVEC and HOTMEC were identical and amounted to 49,000 and those of PAI in cell lysates 52,000 (Fig 3). In cell lysates, a second band with a mol wt of 31,000 was identified.

vWF. Although vWF was demonstrated in both cell types by immunofluorescence (data not shown), vWF could be measured quantitatively only in HUVEC (Fig 4). In ECCM of HUVEC, vWF amounted to $29.2 \pm 12.3 \times 10^{-3}$

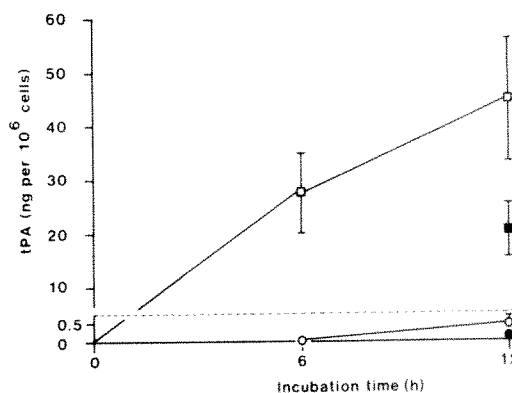


Fig 1. tPA antigen levels (mean $\pm$ SD, $n = 8$) in serum-free conditioned medium of HOTMEC ($\square$) and HUVEC ($\circ$) after six and 12 hours of incubation. Intracellular levels of tPA antigen (mean $\pm$ SD, $n = 8$) of the same HOTMEC ($\blacksquare$) and HUVEC ($\bullet$) at the end of the 12-hour incubation time are given.

U/ 10^6 cells (mean $\pm$ SD; $n = 8$) after six hours and $42.8 \pm 23.4 \times 10^{-3}$ U/ 10^6 cells after 12 hours' incubation. A total of $30.4 \pm 11.7 \times 10^{-3}$ U/ 10^6 cells were measured within the cells after a 12-hour incubation.

Activation of protein C. The activation of protein C by thrombin on the cell surfaces revealed identical profiles in both cell types (Fig 5).

DISCUSSION

The concentrations and activities of relevant coagulant and fibrinolytic factors synthesized by HOTMEC and HUVEC were compared. Although the cells were grown under identical conditions, marked differences in the content and the release of these factors from the cells into the culture medium were observed.

HOTMEC contained and released markedly more tPA than HUVEC. tPA was found within the cells as well as in ECCM in both cell types in an inactive form, which suggests that tPA was completely complexed to PAI under the conditions of this study. On the other hand, it is known that

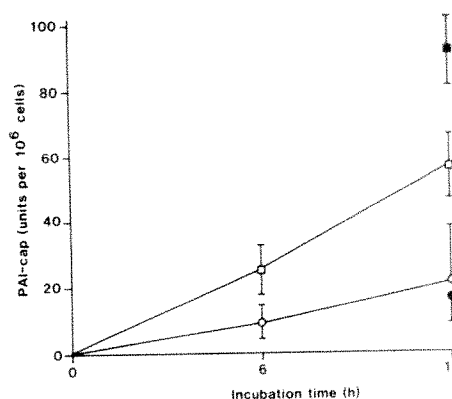


Fig 2. PAI-cap (mean $\pm$ SD, $n = 8$) of serum-free conditioned medium of HOTMEC ($\square$) and HUVEC ($\circ$) after six and 12 hours of incubation. Intracellular PAI-cap (mean $\pm$ SD, $n = 8$) of the same HOTMEC ($\blacksquare$) and HUVEC ($\bullet$) at the end of the 12-hour incubation time are given.

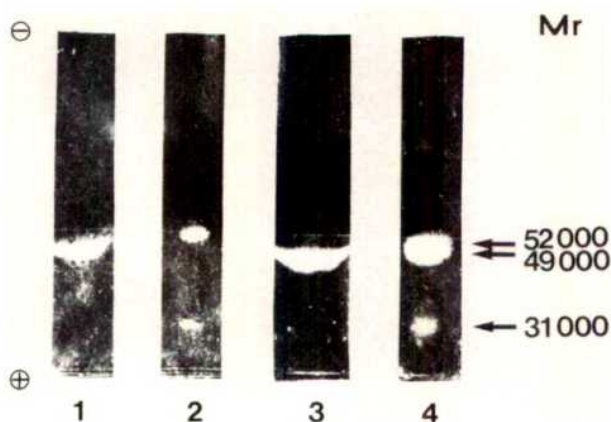


Fig 3. Reverse-phase fibrin autography of cell lysates and ECCM of HOTMEC and HUVEC after incubation of the cells in SFM for 12 hours. Lane 1, ECCM of HUVEC; lane 2, cell lysate of HUVEC; lane 3, ECCM of HOTMEC; lane 4, cell lysate of HOTMEC.

endothelial cells can release an excess of tPA over PAI when they are stimulated, eg, in physical exercise or venous occlusion.¹² The higher amounts of tPA found in HOTMEC may therefore be regarded as a strong potential fibrinolytic capacity.

Active PAI were detected in ECCM and lysates of both cell types by a functional assay as well as by reverse fibrin autography. The high-mol wt PAI in ECCM showed a mol wt approximately 3,000 smaller than that in cell lysates, thus confirming the observation of Kooistra et al¹³ that PAI is degraded to a lower mol wt form after secretion into ECCM. The fact that PAI activity was found also at a mol wt of 31,000 in cell lysates might be due to partial degradation of the 52,000-mol wt molecule during preparation of cell lysates followed by a shift in mol wt as described recently by Levin.¹⁴

vWF was detected in HOTMEC as well as in HUVEC. Obviously, the concentration of vWF is very low in HOT-

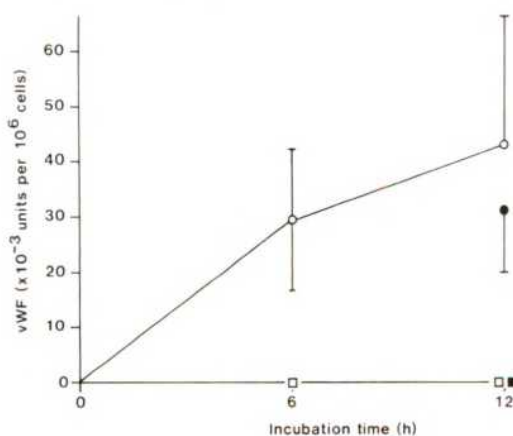


Fig 4. vWF levels (mean $\pm$ SD, $n = 8$) in serum-free conditioned medium of HOTMEC ($\square$) and HUVEC ($\circ$) after six and 12 hours of incubation. Intracellular levels of vWF (mean $\pm$ SD, $n = 8$) of the same HOTMEC ($\blacksquare$) and HUVEC ($\bullet$) at the end of the 12-hour incubation are given.

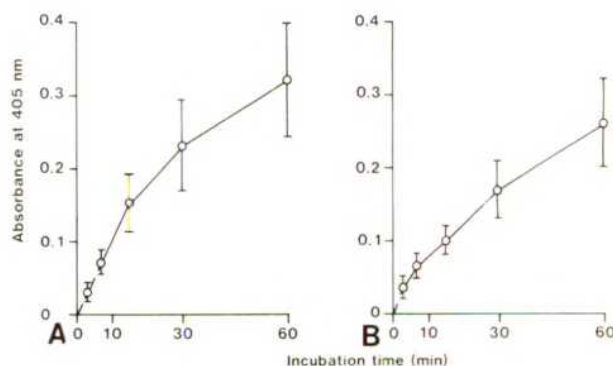


Fig 5. Protein C activation by thrombin on the surface of HUVEC (A) and HOTMEC (B). The cells were preincubated with 45 nmol/L purified human α -thrombin for 15 minutes at 37°C. After the removal of thrombin, 65 nmol/L purified human protein C was applied, and the activity (mean $\pm$ SD, $n = 4$) of the generated activated protein C in the supernatant was measured after different incubation times with the chromogenic substrate S-2266 (Kabi Vitrum AB, Stockholm).

MEC because it could be quantitatively determined by ELISA only in HUVEC. Limited amounts of vWF in microvascular endothelial cells may be of importance in a well-balanced system that provides enough vWF for platelet adhesion in the case of microvessel injury but prevents microthrombosis.

Protein C was activated by thrombin on the surface of both cell types to a similar degree, which suggests the existence of equal numbers of thrombomodulin receptors on the surface of HOTMEC and HUVEC. Considering the relatively large surface made available by the endothelial cells in the microvasculature in relation to the blood volume, a high anticoagulant capacity mediated by protein C activation can be assumed in the microcirculation compared with large vessels.

The present study demonstrates that endothelial cells isolated from different vascular sources provide quantitatively different fibrinolytic and anticoagulant capacities. These quantitative differences may be of importance to maintain a free and constant blood flow. The low incidence of capillary microthrombosis compared with large-vessel thrombosis may be explained by the high fibrinolytic and anticoagulant activities in the microcirculation.

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T Cell γ Gene Rearrangements in Hematologic Neoplasms

By Norio Asou, Masao Matsuoka, Toshio Hattori, Fumio Kawano, Shuichiro Maeda, Kazunori Shimada, and Kiyoshi Takatsuki

Rearrangements of the T cell γ ($T\gamma$) gene were studied in primary neoplastic cells from 75 patients with leukemia or lymphoma. $T\gamma$ gene rearrangements were observed in 19 of 21 T cell neoplasms; 14 of 21 immature B cell leukemias, including 4 out of 5 patients with rearrangements of both immunoglobulin heavy-chain (JH) and T cell receptor β chain ($T\beta$) genes; none out of 14 B cell neoplasms; all 3 unclassified neoplasms; and none out of 16 nonlymphoid leukemias. Thus, $T\gamma$ gene rearrangement is frequently found in immature B cells and is not always found in T cells showing $T\beta$ gene rearrangement, but it is not detected in

nonlymphoid cells. Furthermore, $T\gamma$ gene rearrangement in cells with the germline configuration of the JH and $T\beta$ genes was observed. These results indicate that the detection of $T\gamma$ gene rearrangement does not allow a clear assignment to a particular lineage. However, an analysis of $T\gamma$ gene rearrangement provides a further potential tool to establish the lymphoid cellular origin and clonality of hematologic neoplasms and identify the normal stages of lymphocyte differentiation.

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LYMPHOCYTES are known to express antigen recognition molecules, immunoglobulin (Ig) in B cells, and T cell receptors (TCR) in T cells. Genes for Ig and TCR share certain homologies including similar nucleotide segments consisting of variable (V), diversity (D), joining (J), and constant (C) regions and rearrangements to become functionally active during early B or T cell development, respectively.^{1,2} Therefore, an analysis of the Ig and TCR β chain ($T\beta$) gene rearrangements has become a useful tool for determining the lineage and clonality of lymphoid neoplasms.³⁻⁵

A third gene that rearranges in T cells, designated the T cell γ ($T\gamma$) gene, also exhibits sequence similarity with Ig gene segments and undergoes rearrangement in both suppressor/cytotoxic and helper T cells.⁶⁻⁹ However, the product of the $T\gamma$ gene has not yet been identified, and its precise function remains uncertain. One approach to understanding the function of this molecule is to characterize the diversity of the assembled and expressed $T\gamma$ gene in various cell types including neoplastic cells. In addition, it is of importance to determine whether $T\gamma$ gene rearrangements provide insight into the cellular origin and clonality of lymphoid neoplasms. To our knowledge, $T\gamma$ gene rearrangements in non-T cells have been reported only by Griesser et al¹⁰ who detected rearrangements of the $T\gamma$ gene in six out of seven B cell lymphomas. In these respects, rearrangements of Ig heavy-chain (JH), $T\beta$, and $T\gamma$ genes were studied in 35 B lineage

neoplasms, 21 T cell neoplasms, 3 unclassified neoplasms, and 16 nonlymphoid neoplasms.

MATERIALS AND METHODS

Patients and cell preparations. Twenty-one patients with immature B cell acute lymphoblastic leukemia (ALL), 14 patients with mature B cell neoplasm (10 non-Hodgkin's lymphomas [NHL], and four chronic lymphocytic leukemias [CLL]), 21 patients with T cell neoplasm (two ALL, eight NHL, nine CLL and two adult T cell leukemias), three patients with unclassified ALL, and 16 patients with acute nonlymphocytic leukemia were studied. Mononuclear cells were separated from heparinized peripheral blood or bone marrow by Ficoll-Hypaque density gradient centrifugation, or single-cell suspensions of lymph nodes were obtained from these patients, either at the time of diagnosis or relapse. The specimens of bone marrow, peripheral blood, and lymph nodes were gathered after informed consent was obtained according to the guidelines of the Kumamoto University Medical School Committee for Protection of Human Subjects.

Phenotypic analysis. Surface Ig (sIg) and cytoplasmic Ig (cIg) were detected with fluorescein isothiocyanate-conjugated goat anti-human polyvalent Ig, μ , γ , κ , and λ chains. The expression of common ALL antigen, B, T, and myeloid cell-associated antigen was determined by indirect immunofluorescence using a panel of murine monoclonal antibodies (mAbs): J5 (CD10), B4 (CD19), B1 (CD20), OKT3 (CD3), OKT4 (CD4), OKT6 (CD1), OKT8 (CD8), OKT11 (CD2), Tp40 (CD7), MCS-1 (CDw15), MCS-2 (CDw13), and antiplatelet glycoprotein IIb/IIIa complex.

Southern hybridization analysis.¹¹ Ten micrograms of DNA extracted from mononuclear cells were digested with an appropriate restriction enzyme, size-fractionated by 0.7% agarose gel electrophoresis, and transferred to a nitrocellulose filter. Filter-bound DNA fragments were then hybridized to nick-translated ³²P-labeled probes, washed with appropriate stringency, and visualized on autoradiograms. Rearrangements of the $T\gamma$ gene were examined with a 0.7-kilobase (kb) germline *Hind*III-to-*Eco*RI fragment containing the first J region (J γ 1) of the human $T\gamma$ gene, which was highly homologous with the second J region (J γ 2).⁷

RESULTS

The results of the Southern hybridization analysis with the JH, $T\beta$, and $T\gamma$ gene probes are summarized in Table 1, and representative autoradiograms are shown in Fig 1. The J γ 1 and J γ 2 genes exhibit rearrangement when DNA is digested with *Eco*RI⁹ or *Hind*III.⁷ Cells displaying the germline $T\gamma$

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Table 1. Structure of Rearranging Genes in Hematologic Neoplasms

Cell Type	No. of Cases	Germline	JH	T β	T γ	JH + T β	JH + T γ	T β + T γ	JH + T β + T γ
Immature B	21		6			1	10		4
B	14		13			1			
T	21			2				19	
Unclassified	3				1		1	1	
Nonlymphoid	16	16							

gene with *Eco*RI-digested DNA show either two 2.2-kb *Hind*III restriction fragments containing the J γ 1 and J γ 2 genes or this fragment together with a 5.2-kb fragment containing the J γ 2 gene. This is considered to reflect the existence of a nonallelic polymorphic fragment without a *Hind*III site corresponding to the 5' end of the J γ 2 gene, as previously described by Lefranc and Rabbitts.⁷ The results obtained with *Eco*RI-(Fig 1A) and *Hind*III-digested DNA (Fig 1B) hybridizing to the J γ 1 probe were identical except for the aforementioned polymorphic bands, thereby indicating that these rearrangements could not be ascribed to a restriction fragment length polymorphism. T γ gene rearrangements were detected in 19 of 21 cases of T cell neoplasm; 14 of 21 cases of immature B cell leukemia in which the cells expressed B4, J5, B1 antigen, or cIg but not sIg; none of 14 cases of B cell neoplasm in which the cells expressed a single Ig light-chain isotype, κ or λ ; all three cases of unclassified leukemia in which the cells reacted with neither myeloperoxidase staining nor B4, B1, Tp40, MCS-2, and antiplatelet IIb/IIIa mAb; and none of 16 cases of nonlymphoid leukemia. From 49 different rearranged T γ bands in 36 cases, only nine unique rearrangements were noted, which suggests that the diversity of human T γ genes produced by this recombination mechanism is more limited than that of the Ig or TCR genes.

DISCUSSION

T γ gene rearrangements were observed in 19 of 21 T cell neoplasms, 14 of 21 immature B cell leukemias, and all three unclassified leukemias. Thus, T γ gene rearrangement is not specific for T cells. Rearrangements of both JH and T β genes were also detected in immature B cells from five of 21 patients with ALL, as reported in previous papers.^{4,5,12} It is unlikely that these neoplasms are composed of a biclonal mixture of B and T cells because these neoplastic cells retained a pure immature B cell phenotype. The possibility that bigenotypic cells represent a consequence of neoplastic transformation is also unlikely because of the presence of these cells during normal lymphocyte differentiation.¹³ Therefore, bigenotypic cells were considered to reflect either the differentiation stage at which a committed B or T cell retains the potential to switch to the other lineage or the presence of common molecular mechanisms in rearrangements of JH and T β genes.¹² These possibilities are supported by the existence of similar structural configurations¹⁴ and a common recombinase¹⁵ for the JH and T β genes. T γ gene rearrangements in immature B cells may also be explained by these mechanisms. In immature B cells, however, T γ gene rearrangements were more frequently observed than T β gene rearrangements. In mouse hybridomas derived from fetal liver and fetal thymus, T γ gene rearrangements were detected in the fetal liver cells as well as in the thymus, although T β and then TCR α chain (T α) gene rearrangements occurred primarily or exclusively in the thymus.¹⁶ Therefore, T γ gene rearrangements are suggested to occur at high frequency in early lymphocyte differentiation. On the other hand, none of 14 B cell neoplasms had T γ gene rearrangements, which suggests that immature B cells showing T γ gene rearrangements were blocked in further differentiation. This result is in disagreement with the report of Griesser et al¹⁰ who showed T γ gene rearrangements in six of seven B cell lymphomas. The reasons for the discrepancies between our results and theirs are not clear. Furthermore, none of 16 nonlymphoid leukemias had T γ gene rearrangements. Although the function of the T γ gene product remains unknown, T γ mRNA expression in mice is specifically the highest early in thymic ontogeny and declines thereafter, which suggests that the putative T γ polypeptide plays a role for early T cell differentiation in the thymus.^{16,17} Therefore, functional T γ gene rearrangement and expression may be associated with T cell commitment and involved in the activation of T β gene rearrangement in the same way that productive Ig heavy-chain gene rearrangement activates κ light-chain gene rearrangement.³ This hypothesis is supported by the result that cells from four of five immature B cell leukemias displaying T β gene rearrangement also exhibited T γ gene rearrangement.

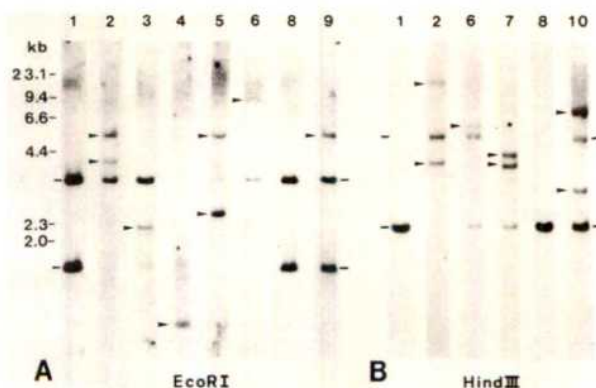


Fig 1. Southern blotting hybridization of the J γ 1 probe to *Eco*RI-(a) or *Hind*III-digested DNA (b) from hematologic neoplasms. Lane 1: DNA of HL-60 cells served as a control for the germline configuration. Lanes 2 through 7: DNAs prepared from immature B cell ALLs. Lane 8: T cell ALL. Lanes 9 and 10: unclassified ALL. DNAs from the same patients are in identical lane numbers in a and b. Dashes indicate the germline 3.5-kb and 1.5-kb *Eco*RI fragments containing the J γ 2 and J γ 1 genes, respectively, and show the two 2.2-kb *Hind*III fragments containing the J γ 1 and J γ 2 genes and a 5.2-kb *Hind*III fragment corresponding to the nonallelic polymorphic fragment of the J γ 2 gene. Arrows on the left of each lane indicate rearranged bands.

However, T γ gene rearrangement was not always found in T cell neoplasms that had T β gene rearrangements; in two of 21 T cell neoplasms, the T γ gene had the germline configuration. This is in contrast with previous reports⁷⁻¹⁰ that T γ gene rearrangements had been found in all cases of T cell neoplasms showing T β gene rearrangements. One possible explanation for our results is that a novel J region of the T γ gene, which is not highly homologous with the J γ 1 or J γ 2 region and does not hybridize to the J γ 1 probe, is used in V-J joining in such T cells. However, Lefranc and Rabbitts⁷ observed T γ gene rearrangements in all 16 T cell lines and 16 cases of fresh T cell leukemia tested using the J γ 1 probe. Quertermous et al⁹ also detected T γ gene rearrangement using the J γ 2 probe in all nine T cell lines and nine T cell neoplasms. Our findings indicate that T γ gene rearrangement and expression may not always be necessary for differentiation and antigen-specific functions of small numbers of T cells.

On the other hand, T γ gene rearrangement associated with the germline JH and T β genes was observed in one unclassified neoplasm as well as in two cases of Ki 1⁺ lymphoma reported by Griesser et al.¹⁰ We also detected this genotype in two cases of NHL (unpublished observation, August 1986). The existence of this novel T cell subgroup is

supported by the findings of Brenner et al¹⁸ who have reported that a small number of normal T3⁺ cells were WT-31⁻, which reacts with a common epitope of human TCR $\alpha\beta$ chains, and expressed T γ mRNA but not TCR $\alpha\beta$ mRNA, although most T3⁺ cells were WT-31⁺ and expressed TCR $\alpha\beta$ and T γ mRNA.

Consequently, T γ gene rearrangement is not specific for T cells and not always found in T cells but is largely restricted within lymphoid cells. Furthermore, the existence of cells showing T γ gene rearrangement but with the germline configuration of the JH and T β genes was demonstrated. Therefore, an analysis of T γ gene rearrangement provides a further potential tool for establishing the lymphoid cellular origin and clonality of hematologic neoplasms. Such studies would be useful not only in the diagnosis and evaluation of the clinical features and prognosis of patients with lymphoid neoplasm but also as a framework to identify normal stages of lymphocyte differentiation.

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CONCISE REPORT

bcr-abl RNA in Patients With Chronic Myelogenous Leukemia

By E. Shtivelman, R.P. Gale, O. Dreazen, A. Berrebi, R. Zaizov, I. Kubonishi, I. Miyoshi, and E. Canaani

The major consequence of the formation of the Philadelphia (Ph¹) chromosome characteristic of leukemia cells of patients with chronic myelogenous leukemia (CML) is fusion of *c-abl* and *bcr* genes. Using a sensitive RNase protection technique, we analyzed mRNA from a large number of CML patients. In most, we identified one or both species of *bcr-abl* chimeric transcripts. These two mRNAs

vary in the specific *bcr* exon joined to *abl* exon II and are translated into slightly different proteins. The amounts of the fused mRNA within leukemia cells vary considerably between individuals and do not correlate with the phase of the disease.

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CHRONIC MYELOGENOUS LEUKEMIA (CML) is characterized by two major phases. The chronic phase lasts an average of 3 years and is characterized primarily by accumulation of granulocytes and their precursors in the bone marrow and peripheral blood. This is followed by the acute phase (blast crisis) in which the leukemia cells fail to differentiate and to respond to regulatory factors of myelopoiesis. In some patients there is an intermediate accelerated phase.¹

A specific chromosomal abnormality, the Philadelphia chromosome (Ph¹), is present in 90% to 95% of CML patients.² This aberration results from a reciprocal translocation between chromosomes 9 and 22 designated t(9;22).³ Recent molecular studies implicate the *abl* oncogene and the *bcr* gene in CML. As a result of the translocation, *abl* is moved from chromosome 9 into the *bcr* gene on chromosome 22.⁴ The fused *bcr-abl* gene is transcribed into a large chimeric RNA which presumably initiates at the *bcr* promoter and is composed of *bcr* sequences 5' of the translocation point on chromosome 22 and sequences of chromosome 9 including *abl*, located downstream of the translocation point.^{5,6} The primary transcript is spliced into a mature *bcr-abl* mRNA of 8 kb.^{7,8,9} This mRNA is translated into a 210-kd protein unique to CML cells,^{10,11} and possessing enhanced tyrosine kinase activity compared to the normal *abl* protein.¹²

In a previous study⁵ we reported that in the 8-kb RNAs of two CML cell lines (K562 and EM2) the junction point is composed of *abl* exon II and *bcr* exon "3" sequences. We now sought to characterize the RNA junction in CML patients. In addition, we inquired whether the transition from the chronic to the acute phase of the disease involves increased transcription of the fused *bcr-abl* gene.

MATERIALS AND METHODS

Leukemia cells were isolated from the buffy coat of peripheral blood. RNA was prepared using the urea/lithium chloride method.¹³ Polyadenylated messenger RNA was isolated by purification on oligo-dT-cellulose columns. RNase protection analysis for the alternative *bcr-abl* junctions has been described.¹⁴ RNA probes were synthesized by standard techniques.¹⁵

RESULTS

For qualitative and quantitative analyses of *bcr-abl* RNA junctions in CML patients, we used the RNase protection technique. For probes we used two different junctions derived from cDNA clones K-28 and L-6. In the K-28 clone *abl* exon II is joined to *bcr* exon "3"; in L-6, *abl* exon II is

linked to *bcr* exon "2" (enumeration of *bcr* exons is according to ref 16). The positions of the relevant *abl* and *bcr* exons on the Philadelphia chromosome are shown in Fig 1, and the structure of the two probes is shown in Fig 2. RNAs of CML patients were annealed to each of the two probes and following RNase digestion were electrophoretically analyzed. Examples of these analyses are shown in Fig 2. For example, RNA from patient S-15 (Fig 2A, lane 1) protected the full-length 450-b K-28 probe. This indicates that within the 8-kb RNA of this patient, *abl* exon II is linked to *bcr* exon "3". The additional bands apparent in the autoradiogram result from protection of the 278-b *abl* segment within the probe by normal *abl* RNA, and from protection of the 172-b *bcr* DNA in the probe by normal *bcr* RNA. Hybridization of the L-6 probe to RNA from patient 1380 (Fig 2B, lane 1) resulted in protection of the 280-b probe. This implies that the *bcr-abl* RNA junction of this patient is composed of *abl* exon II and *bcr* exon "2" sequences. Bands of 250 and 255 b seen in this experiment represent protection of segments of the probe by normal *abl* RNAs containing alternative first exons.¹⁴

Results of analyses of samples from 21 CML patients are shown in Table 1. RNAs of 19 patients contained one or both RNA junctions. In two patients with Ph¹ chromosome we could not detect either junction. Seven patients had only the K-28 junction, and four only the L-6 linkage. Surprisingly, eight patients showed both junctions.

We next questioned whether the progression of the disease

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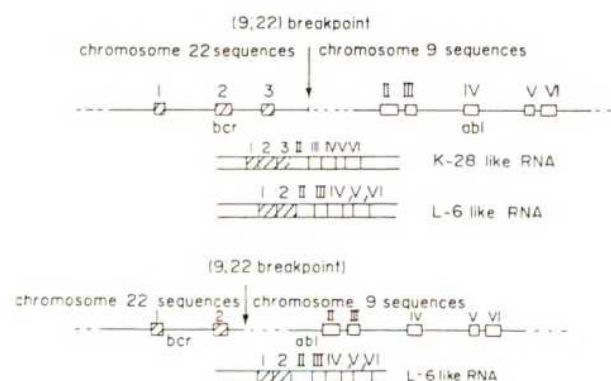


Fig 1. Formation of RNAs with a particular *bcr-abl* junction point depends on the chromosomal breakpoint and on alternative splicing. Hatched boxes represent *bcr* exons and are arbitrarily enumerated according to ref 15. Open boxes represent *abl* exons.⁵ The L-6 RNA junction at the top is formed by a mechanism of alternative splicing involving skipping of *bcr* exons "3".

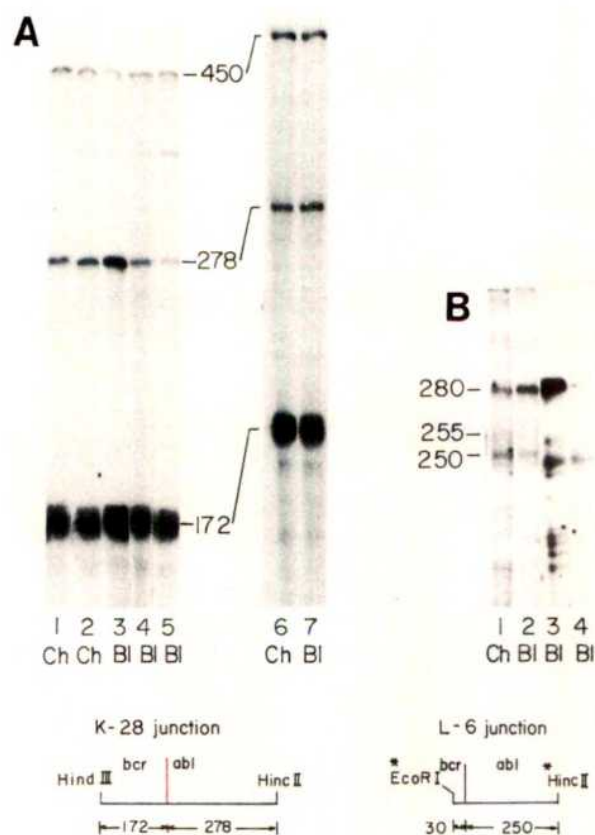


Fig 2. *bcr-abl* RNA junctions in CML patients. (A) Protection of the K-28 junction by RNAs from patients S-15, S-10, 9081, 1266, 9037, 1158, and 1158 (lanes 1, 2, 3, 4, 5, 6, and 7, respectively). The last two samples were analyzed on a separate gel. L-6 junction was not detected in these patients. (B) Protection of the L-6 junction by RNAs from patients 1380, 1176, 9033, and 9011 (lanes 1, 2, 3, and 4, respectively). K-28 junction was not detected in these patients. Ch and Bl designate patients in chronic phase or blast crisis, respectively. *EcoRI is a linker site. *HincII site was shifted 20 nucleotides to the 5' because of a small deletion in L-6.

Table 1. Qualitative and Quantitative Analysis of *bcr-abl* RNA in CML Patients

UPN	Ph ¹	Phase	Junction*	
			K-28	L-6
S-10	+	chronic	3	—
S-15	+	chronic	4	—
1380	+	chronic	—†	3
1265	+	chronic	1	4
1256	+	chronic	3	2
9058	+	chronic	10	10
1373	+	chronic	2	3
1263	+	chronic	10	4
1056	+	chronic	3	3
1269	+	chronic	—	—
1158	+	chronic	4	—
1249	+	chronic	1	2
9037	+	acute	3	—
1176	+	acute	—	5
9033	+	acute	—	10
9011	+	acute	—	1
1266	+	acute	3	—
9031	+	acute	7	2
1267	+	acute	—	—
1158	+	acute	5	—
9081	+	acute	2	—

*Relative amount of fused *bcr-abl* RNA ranked in a scale of 1 to 10 by signal intensity.

†No signal detected.

from chronic to acute phase is associated with enhanced synthesis of the fused *bcr-abl* RNA. We compared the signal intensity of the 450-b K-28 probe protected by RNAs from two patients in chronic phase (Fig 2A, lanes 1 and 2) and by RNAs from three patients in acute phase (blast crisis) (Fig 2A, lanes 3, 4, and 5). No correlation between signal intensity and disease phase was detected. Moreover, samples from the same patient taken during both phases of the disease contained similar amounts of the fused RNA (Fig 2A, lanes 6 and 7). Similar analyses were performed with RNA samples from patients with only the L-6 junction (Fig 2B). Again, we observed variability in the amounts of the fused RNAs and lack of correlation with disease stage. Quantitative results of these analyses are shown in Table 1.

DISCUSSION

In this study we demonstrate that RNA from 19 of 21 CML patients contain one or both of two alternative *bcr-abl* RNA junctions transcribed from the chimeric *bcr-abl* gene formed on the Ph¹-chromosome. In two patients with the Ph¹-chromosome, neither junction was detected. Levels in these individuals may have been below detection. Alternatively, these individuals may have fused RNAs in which different *abl* and *bcr* exons constitute the junction point. The resolution of which *bcr* exon will constitute the RNA junction point is most simply determined by the position of the t(9;22) translocation point on chromosome 22. Thus, if the breakpoint is between *bcr* exons "3" and "4," *abl* exon II will be linked to *bcr* exon "3." If the breakpoint occurs between *bcr* exons "2" and "3," the RNA junction point will be derived from *abl* exon II and *bcr* exon "2" (Fig 1). The

surprising finding that a large proportion of patients contain both RNA junctions probably represents alternative RNA splicing; when the breakpoint is between *bcr* exons "3" and "4," *abl* exon II can alternatively splice to *bcr* exons "3" and "2" (and possibly to other upstream *bcr* exons) to give two different RNA junctions (Fig 1). The two RNAs produced will be translated into proteins which vary in the 25 amino acid peptide coded by *bcr* exon "3."¹⁶ Whether the two proteins possess different biological activity is unknown. Interestingly, the breakpoint in 15 of 19 (79%) cases occurred between *bcr* exons "3" and "4".

In the second part of this work we showed that the amount of *bcr-abl* mRNA varies considerably between patients. Moreover, cells from patients in the acute phase do not contain, on average, higher levels of the fused *bcr-abl*

transcript. On the basis of these data, we conclude that transition from chronic to acute phase of CML is not the result of increased expression of the *bcr-abl* gene. Our studies do not exclude qualitative and quantitative alterations in the fused protein during phase transition. However, it is likely that other genetic changes, such as those associated with the additional chromosomal alterations typical of CML in acute phase,¹⁷ underlie the phase transition of the disease.

ACKNOWLEDGMENT

We thank Drs J. M. Goldman, E. Aghai, E. Januscewicz, M. Prokocimer, and T. Umiel for clinical samples. E. Canaani is a recipient of a Career Development Award from the Israel Cancer Research Fund and holds the Harry Kay Professorial Chair in Cancer Research.

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CORRESPONDENCE

COBALAMIN-FOLATE INTERRELATIONS

To the Editor:

I would like to comment on two points made by Dr Chanarin and his colleagues in their critical and comprehensive review of cobalamin-folate interrelations.¹

First, they state that "megaloblastic erythropoiesis is unique to humans." They have overlooked completely the classic writings of Marston, who in 1952 described a profound macrocytic anemia in which the oxygen-carrying capacity fell to 30% or lower in sheep suffering from cobalt deficiency.² Two further features were subsequently added to the picture: (1) the presence of abnormal primitive erythroblasts in the peripheral blood and (2) a spectacular response to folic acid. These were observed both in sheep reared on cobalt-deficient pastures and in experimental sheep rendered vitamin B₁₂ deficient under carefully controlled laboratory conditions. In these animals "the blood picture proclaims a disorder of bone marrow very similar to that which is evident in pernicious anemia and like the latter responds dramatically to folic acid."³ In a further publication Marston described the anemia as megaloblastic and noted that it responds equally dramatically to vitamin B₁₂ as well as to folic acid.⁴ True, the sheep is not the ideal experimental animal in which to study megaloblastic anemia because in it the effects of adenocobalamin deficiency are usually much more profound than those of methylcobalamin deficiency. It succumbs to the former usually before any serious degree of anemia develops.

Second, the authors state that "there is an unconfirmed report of increased urinary excretion of methylmalonic acid in patients anesthetized with N₂O."⁵ Other important substances are metabolized along the propionyl-succinyl pathway. Notable among these are valine and isoleucine. Dietary loading with both these amino acids will increase the methylmalonic aciduria in untreated pernicious anemia, indicating that their metabolism also is impaired at the methylmalonyl-CoA mutase block.^{6,7} This is true also in patients under nitrous oxide anesthesia. Serum valine levels were significantly elevated in patients anesthetized with nitrous oxide for periods of three hours or longer. This was attributed to the inactivation of adenosylcobalamin by nitrous oxide, thereby inducing the methylmalonyl-CoA mutase block with resulting accumulation of valine in the blood.^{8,9} The urinary excretion of methylmalonic acid was not increased in these patients (unpublished observation). Isoleucine, which is only partly metabolized along the propionyl-succinyl pathway, as well as valine, and partly along a vitamin B₁₂ independent ketogenic pathway, can also be elevated in patients undergoing nitrous oxide anesthesia, but the serum levels of both these amino acids show a pronounced fall in patients anesthetised without nitrous oxide.¹⁰ Three metabolites, valine, isoleucine, and methylmalonic acid, therefore accumulate proximal to the methylmalonyl-CoA mutase block in humans exposed to nitrous oxide.

This is convincing evidence that, unlike in the rat, the enzyme methylmalonyl-CoA mutase is inactivated by nitrous oxide in man. The serum valine levels measured by the highly sensitive microbiological method employed¹¹ (mean recovery 99.4%, coefficient of variation $\pm 3.2\%$ in 12 experiments after adding 4.0 mg % of L-valine to normal serum) would appear to be a more sensitive indicator of the mutase activity than the urinary methylmalonic acid under these very short periods of exposure to nitrous oxide.

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To the Editor:

In response to Dr T.E. Parry, the anemia in sheep reared on cobalt-deficient feed and hence developing cobalamin deficiency when examined by hematologists was reported to be normocytic and normochromic,¹ and the marrow was hypoplastic.² The assumption 30 years ago that the anemia in sheep was megaloblastic was not based on any supporting evidence.

We are not aware of any convincing evidence for the inactivation

of methylmalonyl-CoA mutase by short-term exposure to nitrous oxide in man.

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CLONAL ORIGIN OF ERYTHROID CELLS IN JUVENILE CHRONIC MYELOGENOUS LEUKEMIA

To the Editor:

We read with interest the article "Juvenile Chronic Myelogenous Leukemia: Characterization of the Disease Using Cell Cultures" by Estrov et al that appeared in a recent issue of this journal.¹

Our observations with an *in vitro* progenitor cell assay in two patients with juvenile chronic myelogenous leukemia (JCML) led us to different conclusions. Namely, the erythroid cells in this disease also descend from the leukemic clone. The erythroid cells from our patients proliferated *in vitro* and formed BFU-E colonies that could not be morphologically distinguished from BFU-E colonies of normal marrows, but they exhibited the karyotype of the leukemic clone. Thus JCML appears to be a multipotent stem cell disease like the Philadelphia chromosome-positive CML. Sato et al² also alluded to the fact that their observations of JCML suggested the pluripotent stem cell origin of JCML.

In our patients (see Table 1 for the summary of our clinical and laboratory findings), the bone marrow and blood specimens formed erythroid colonies. We used the culture method described by Ogawa et al³ for erythroid colonies in case 1 and the CFU-GEMM culture method described by Messner and Fauser⁴ with some modification in case 2. The modification consisted of using 30% plasma-derived horse serum (Sterile Systems, Inc., Logan, UT) and conditioned medium derived from culture of the human bladder carcinoma cell line 5637⁵ instead of human plasma and phytohemagglutinin LCM, respectively.

In case 2, the initial fresh marrow sample yielded two cytogenetically distinct clones, one with 45,XY,-7 (29 of 32 cells) and the

other, 46,XY (three of 32 cells). The results of CFU-GM and CFU-GEMM cultures are shown in Table 1. We cytogenetically examined pooled colonies that formed in the plate from the CFU-GEMM culture. Of 75 metaphases analyzed, 73 were 45,-7. One was 46,XY, and the other had 90 chromosomes. The blood cells cultured with phytohemagglutinin for three days yielded only the normal karyotype (26 metaphases examined). After the patient received one course of daily VP-16 and cyclophosphamide for five days, a blood sample was again cultured for CFU-GEMM. The sample formed an average of 221 colonies (duplicate plates) consisting of 43 erythroid, 33.5 mixed erythroid and granulocyte-macrophage, 98.5 pure macrophage, and 46 granulocyte-macrophage. The granulocyte or macrophage differentiation was confirmed by naphthol-ASD chloroacetate and α -naphthyl acetate esterase stains using Sigma cytochemical kits (catalogue no. 90-C2 and 90 A-1, Sigma Chemical Co, St Louis). All colonies formed in one dish were pooled and prepared for cytogenetic analysis. Separately, well hemoglobinized erythroid colonies from another dish were picked, pooled, and prepared for cytogenetic analysis. Both preparations yielded only metaphases with 45,XY,-7 (15 metaphases were examined from each preparation). The results strongly suggested that the erythroid cells derived from the leukemic clone proliferated *in vitro*. Since the erythroid colonies did not grow in the absence of erythropoietin, their growth appeared to be under the erythropoietin control.

Growth of erythroid colonies from bone marrow cells from a JCML patient has been described.⁶ Investigations of erythroid cells in this disease suggested a number of fetal characteristics such as a high fetal hemoglobin concentration and a prevalence of decreased

Table 1. Summary of Two JCML Cases

Parameter	Case 1	Case 2
Age at diagnosis (mo)	8	8
Hb (g/dL)	9.0	11.6
WBC ($\times 10^{-3}/\mu\text{L}$)	29.0	22.0
Monocytes (%)	29.0	31.0
Platelet ($\times 10^{-3}/\mu\text{L}$)	31.0	67.0
Fetal Hb (%)	3.1	3.1
LAP (15-100)	63	ND
Lysozymes ($\mu\text{g/mL}$) (5-13)	7.8	16.2
Spleen (below costal margin)	8 cm palpable	2 cm palpable
Cutaneous rash	present	present
Bone marrow blasts (%)	10	15
Marrow karyotype	46, XY	45, XY, -7/46, XY
Progenitor cell assay at diagnosis		
Marrow		
CFU-GM/ 5×10^4 (197.0 $\pm$ 111.5)	369 $\pm$ 29	550
CFU-GEMM/ 1×10^5 (173 $\pm$ 101)	ND	315 (59 BFU-E and 11 mixed)
BFU-E/ 5×10^4 (32.7 $\pm$ 20.5)	139	ND
Blood		
CFU-GM/ 5×10^5 (16.4 $\pm$ 8.7)	127 $\pm$ 9	1,670
CFU-GEMM/ 1×10^5 (14.3 $\pm$ 9.4)	ND	342 (96 BFU-E and 30 mixed)
BFU-E/ 5×10^5 (33.5 $\pm$ 6.5)	107 $\pm$ 11	ND

Figures in parentheses indicate the range of the normal sample or the mean $\pm$ 1 SD of control samples.

Abbreviation: ND, not done.

red cell I antigen.^{6,7} Whether this is an indication of the malignant erythroid clone or an epiphenomenon is unknown.

We are uncertain regarding the reasons for the discordant results of ours and those of Estrov et al.

Neither of our patients exhibited a high fetal hemoglobin concentration. It is possible that the high fetal hemoglobin variety may be a disease different from JCML without a high fetal hemoglobin content. This could be answered by culturing marrow and blood cells from patients with and without high fetal hemoglobin concentrations.

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To the Editor:

The letter by Inoue et al provides further insight into the disordered hematopoiesis that characterizes juvenile chronic myelogenous leukemia (JCML). The relevant data are confined to the cytogenetic analysis of the plucked, well-hemoglobinized BFU-E erythroid colonies derived from cultures of the peripheral blood of case 2. The investigators demonstrated nicely that 15 erythroid cells in metaphase at the time of study showed monosomy 7, thus indicating their derivation from the leukemic clone. This does not prove that all of erythropoiesis in vivo and in vitro in case 2 was derived from the malignant clone because not all the BFU-E colonies were plucked for study and not all of the colonies that were selected were composed of cells in metaphase. Nevertheless, the data are important because they identify the leukemic "event" in case 2 at an early point in hematopoiesis, possibly at the level of the pluripotent stem cell similar to Philadelphia chromosome + adult CML.

As Inoue et al indicate, the cell culture findings from both of their patients at diagnosis were different than those that we reported.¹ Using CFU-GEMM and CFU-C assay systems, marrow and peripheral blood from our patients at diagnosis yielded only monocyte-macrophage colonies. Most importantly, the excessive proliferation of these colonies persisted in cultures lacking both adherent cells and exogenous colony-stimulating activity. We regard this latter finding as a diagnostic hallmark of JCML.

Since our publication, we have performed serial CFU-GEMM studies on peripheral blood from two patients who became resistant

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to chemotherapy and developed recurrent, aggressive JCML. Specimens from both of these patients still yielded monocyte-macrophage colonies but also large numbers of highly proliferative BFU-E colonies that continued to grow actively on replating in secondary cultures. Karyotypes on plucked BFU-E colonies from both cases were normal, whereas monocyte-macrophage colony karyotypes were abnormal. These results differ but do not contradict those of Inoue et al for the technical reasons stated before.

The emergence of in vitro erythropoiesis in our cases may indicate that cell culture profiles in JCML change with time, chemotherapy, or advancing disease. Since marrow from both patients of Inoue et al showed excessive numbers of blasts, they may have already been in a highly malignant phase of disease similar to our cases that yielded BFU-E colonies.

Ongoing serial studies by interested workers will hopefully provide an answer to this and other aspects of JCML.

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ERRATUM

In the article by Pui et al entitled "Shifts in Blast Cell Phenotype and Karyotype at Relapse of Childhood Lymphoblastic Leukemia," which appeared in the December 1986 issue, a typesetting error was made in Table 3. The partial karyotype in column 1 for Case 1 is repetitious and should be ignored.

ANNOUNCEMENTS

AMERICAN BOARD OF INTERNAL MEDICINE 1987 SUBSPECIALTY EXAMINATIONS IN MEDICAL ONCOLOGY AND CRITICAL CARE MEDICINE

Registration Period: January 1, 1987 through April 1, 1987
Examination Date: November 10, 1987

For information and applications forms, please contact: Registration Section, American Board of Internal Medicine, 3624 Market Street, Philadelphia, PA 19104.

LEUKEMIA SOCIETY OF AMERICA THIRD ANNUAL NATIONAL SYMPOSIUM

**Town and Country Hotel, San Diego, CA
March 18-21, 1987**

Designed to focus on the latest advances and techniques in research and treatment of leukemias and lymphomas, the program will address current information and rapidly developing new approaches and programs in studies of the disease. Emphasis will be upon the application of basic science and its relation to clinical practice. The meeting is geared to physicians, nurses, allied professionals, fellows-in-training, residents, and students with major interest and involvement in leukemia and related disorders. The course is accredited for CME by the AACME. It meets the criteria for 17 credits in Category I towards the Physician's Recognition of the AMA. Registered nurses will qualify for 20.7 contact hours of continuing education through the LSA's provider accreditation for continuing education for registered nurses.

There will be: a keynote address on "Chromosomes, Genes, and Leukemia" by Peter C. Nowell, MD; Stohlmán Memorial Scholar papers; posters; and five minisymposia, dealing with biological characteristics of clinical trial designs, molecular genetics, application of molecular probes, antimyeloid monoclonal antibodies, and cellular response to growth factors. There will also be daily group breakfasts and an awards banquet. Registration fee: physicians: \$100; nurses, fellows-in-training, residents, and students: \$60. Fees are due by March 11, 1987.

For registration and additional information, contact: LSA Medical Conference, c/o Bostrom Corporation, 435 N Michigan Ave, Suite 1717, Chicago, IL 60611. Telephone: (312) 644-0828.

21ST ANNUAL CONFERENCE ON PRACTICAL, CLINICAL, AND LABORATORY ASPECTS OF BLOOD COAGULATION

April 27-29, 1987

5TH ANNUAL ANTIBODY, SPECIAL COAGULATION, AND PLATELET TECHNIQUES WORKSHOP

April 25-26, 1987

Kellogg Center, Michigan State University

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Course Director: Dr John A. Penner

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For further information, contact: Mary Edna Helfer, Conferences, A118 E Fee Hall, Michigan State University, East Lansing, MI 48824-1022. Telephone: (517) 353-8856.

SYMPOSIUM ON PLASMA CELL MYELOMA AND RELATED DISEASES**April 10, 1987****Duke University Medical Center, Durham, NC****GUEST SPEAKERS**Daniel E. Bergsagel, *Toronto*M. Robert Cooper, *Winston-Salem*Brian G.M. Durie, *Tucson*Thomas A. Waldmann, *Bethesda*T.J. McElwain, *London*Howard Ozer, *Chapel Hill*

This symposium is sponsored by the Hematology/Oncology Division of the Department of Medicine. The natural history, clinical presentation, and evolution of plasma cell myeloma will be reviewed along with staging, treatment, and prognosis using standard regimens. The investigational use of high-dose melphalan chemotherapy and the interferons will be reported, and current investigations regarding the molecular biology of B cell neoplasms. Particular attention will be given to problems and complications encountered in patients with plasma cell myeloma and controversies in treatment. *For further information, write:* Myeloma Symposium, Box 3096, Duke Medical Center, Durham, NC 27710.

DR H. DUBOIS-FERRIÈRE—DINU LIPATTI RESEARCH FELLOWSHIP IN HEMATOLOGY

The Dr H. Dubois-Ferrière—Dinu Lipatti Foundation offers a research fellowship to a medical graduate with at least two years' experience in research. The recipient of the fellowship will have the opportunity to spend two or three years working on a hematology research program in Geneva. The annual stipend ranges from US \$30,000 to \$35,000.

For further information, contact: Fondation Dr H. Dubois-Ferrière—Dinu Lipatti, case postale 225, CH-1227 Carouge-Genève, Switzerland.

XIX MEETING OF THE INTERNATIONAL SOCIETY OF PEDIATRIC ONCOLOGY (SIOP)**September 13–18, 1987****Jerusalem Laromme Hotel, Jerusalem, Israel**

Deadline for abstracts: April 15, 1987

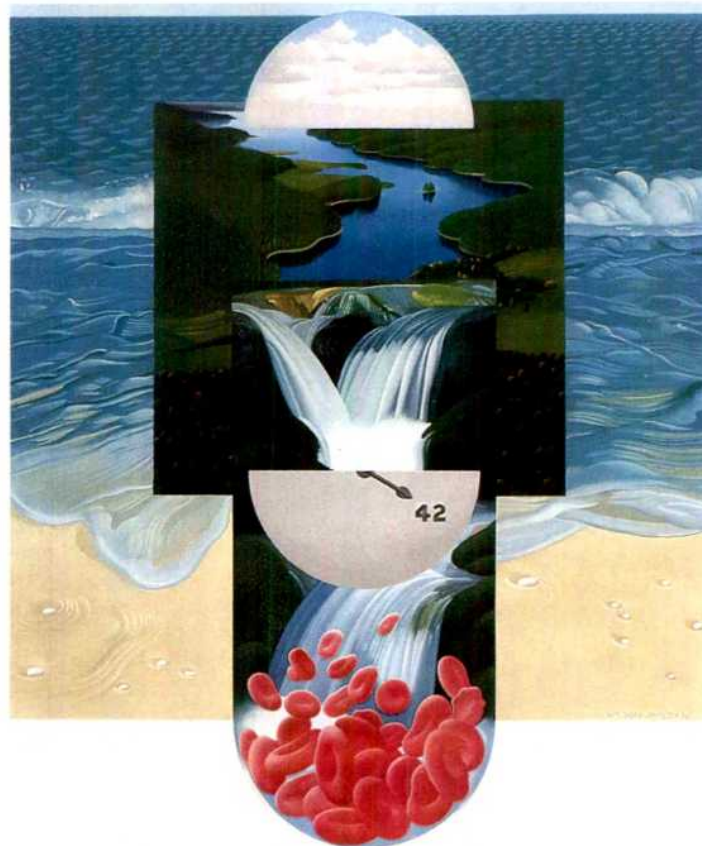
For further information, write: Prof R. Zaizov, PO Box 983, Jerusalem 91009, Israel.

5TH EUROPEAN CONFERENCE ON CLINICAL HEMORHEOLOGY**June 29–30 and July 1, 1987****Bordeaux, France**

The Presidents of the conference will be Prof P. Boivin and Prof J-F. Stoltz. The main topic will be the importance of blood methodology to clinical studies. There will be interdisciplinary sessions organized in collaboration with specialist societies for angiology, hematology, neurology, and thrombotic disorders.

For further information, contact: Prof Michel R. Boisseau, Secretariat Scientifique, Laboratoire d'Hématologie, Hôpital Cardiologique, Avenue Magellan, 33604 Pessac, France. Telephone: 56 36 24 45, extension 33148. Telex: 560 349 F.

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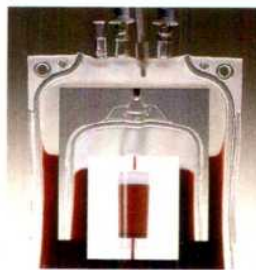


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NEW
JOURNAL
FOR 1987

MOLECULAR AND CELLULAR PROBES

The Location, Diagnosis and Monitoring of Disease by Specific Molecules and Cell Lines

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C. N. Hales

*Department of Clinical Biochemistry,
University of Cambridge,
Cambridge, UK*

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Maryland, USA*

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The journal will publish the description, technical and clinical evaluation of specific molecules and cell lines which may be used in the diagnosis, location and monitoring of disease.

Also to be included are the description of new or greatly improved strategies for the production of molecules or cell lines potentially suitable as probes and of new strategies for the generation and detection of signals from such probes. Animal studies in so far as they are clearly relevant as models or tests of techniques applicable to man also come within the scope of the journal. The journal aims to cross the traditional boundaries of diagnostic and clinical medicine departments since new probes or new approaches to the development of probes are likely to be of interest to workers in a variety of fields outside that of the original discoverers. By the publication of new work and reviews the journal will aim to keep readers in close contact with the most recent advances in this rapidly expanding area of medicine.

Research Areas Include:

- Detection and measurement of specific molecules by polynucleotides, monoclonal antibodies and cloned hybridomas or cell lines.
- Improved strategies for the production of these probes.
- Improved methods for the generation or detection of signals from these probes *in vivo* or *in vitro*.
- Animal models used as tests of the probes.

Manuscripts for Publication to:

Professor C. N. Hales,

*Department of Clinical Biochemistry, University of Cambridge,
Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, UK
(Monoclonal Antibodies and Cell Lines)*

Dr. R. H. Yolken

*Department of Pediatrics, The Johns Hopkins University,
Baltimore, Maryland 212305, USA
(DNA Probes and Infectious Diseases)*

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*Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma.
Ann Intern Med 102:596-602, 1985.

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Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma.
Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and

dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO}

falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

- (1) Maintain FI O₂ at concentrations approximately that of room air (25%) during surgery and the post-operative period.
- (2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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Tibor J. Greenwalt, M.D.
Director, Hoxworth Blood Center
Professor of Internal Medicine
University of Cincinnati
3231 Burnet Avenue
Cincinnati, Ohio 45267

RESEARCH SCIENTIST— CELLULAR IMMUNOLOGY/HEMATOLOGY

The Wadsworth Center for Laboratories and Research is seeking a research scientist with an interest in cellular immunology and hematology. The individual should have experience in flow cytometry and modern hematology instrumentation. The scientist will be encouraged to develop his/her own research interests, as well as to collaborate with other scientists to encourage developing cellular immunology and hematology research. The scientist will also have responsibilities for providing expertise in implementing appropriate proficiency testing programs in flow cytometry and hematology. There is a very definite commitment at the Wadsworth Center for support of innovative research of public health relevance. The candidate must have either a Ph.D., M.D., or D.V.M. with appropriate experience.

Please send CV and the names of three references to: Felix H. Heilpern, Wadsworth Center for Laboratories and Research, Empire State Plaza, Albany, NY 12201. An Affirmative Action/Equal Opportunity Employer.

NON-TENURE TRACK APPOINTMENT

Assistant Professor and Assistant Medical Director, University of Minnesota Hospital, Blood Bank, and St. Paul Region, American Red Cross Blood Services.

A position is available for a physician, Board certified or Board eligible in clinical pathology with blood banking fellowship or one year of experience in clinical pathology beyond residency. Alternatively, the candidate will be board certified in Internal Medicine or Pediatrics and board certified or board eligible in Hematology. Candidates should have experience in transfusion medicine and immunohematology.

This position will be involved in the teaching of blood banking/immunohematology through formal courses, informal supervision, and teaching of medical students, residents, fellows, and allied health students. This position will be expected to participate in basic or clinical research relating to one or more of the following areas: blood collection; platelet serology and transfusion; neutrophil structure, function or serology; apheresis; bone marrow donation or transplantation; histocompatibility; transfusion transmitted diseases; or other blood bank related topics.

This position will serve as Assistant Medical Director of the Regional Blood Services and the University Hospital Blood Bank and will assume partial responsibility for the daily operation of these facilities. This may include responsibility for the general blood bank laboratory.

This position is a temporary, non-tenure track faculty appointment which involves major service commitments. Applicants should send their curriculum vitae and three letters of recommendations to:

Richard Brunning, M.D.
Box 198 UMHC
Department of Laboratory Medicine and Pathology
University of Minnesota Hospital and Clinic
420 Delaware Street S.E.
Minneapolis, MN 55455

Deadline for receipt of applications is March 31, 1987. The University of Minnesota is an equal opportunity educator and employer and specifically invites and encourages applications from women and minorities.

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There will be a contributed poster session. The deadline for submission of abstracts is **June 1, 1987**. A 200-word summary of work to be presented should be sent to: **Frederick A. Ofsu, Ph.D., Department of Pathology, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5.**

Conference Chairs:

Frederick A. Ofsu, Ph.D., Department of Pathology

Jack Hirsh, M.D., Department of Medicine

McMaster University, Hamilton, Ontario, Canada
and

**Isidore Danishefsky, Ph.D., New York Medical College, Valhalla,
New York**

For program and registration information: Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021.

**ASSISTANT DIRECTOR,
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The Division of Hematology of the Department of Medicine, Stanford University School of Medicine, invites applications for Assistant Director of the newly formed Bone Marrow Transplant Unit. The position carries the rank of Assistant Professor of Medicine in the Division of Hematology. The faculty person will interact with clinicians and investigators in the Departments of Pediatrics, Pathology and Radiation Therapy, as well as the newly formed Center for Molecular and Genetic Medicine. Candidates should have experience and be expert in allogeneic and autologous Bone Marrow Transplantation. In addition, it is anticipated that the faculty person holding this position will devote approximately 60% of time to intensive work in the areas comprising transplantation biology. Thus, candidates should have demonstrated ability to develop an independent, self-sustaining research program.

Applicants should send a curriculum vitae, a brief statement of their research interests and names of two references to: **Stanley L. Schrier, M.D., Chief, Division of Hematology, Stanford University School of Medicine, Stanford, California 94305.**

Stanford University is committed to increasing representation of women and members of minority groups on its faculty and particularly encourages applications from such candidates.

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The Cooley's Anemia Foundation, Inc., invites applications for fellowships to both postdoctoral fellows and junior faculty members interested in the field of Cooley's anemia with stipends of up to \$15,000. The deadline for receipt of applications is April 1, 1987. Awards will be made by June 15, 1987, and funding will begin July 1, 1987, for one year with the possibility of renewal for a second year.

For application forms and further information, write: **Mr. Michael C. DiFilippo, National Executive Director, Cooley's Anemia Foundation, Inc., 105 East 22nd Street, Suite 911, New York, New York 10010. Telephone: (212) 598-0911.**

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NON-TENURE TRACK APPOINTMENT

Assistant Professor and Assistant Medical Director, University of Minnesota Hospital, Blood Bank, and St. Paul Region, American Red Cross Blood Services.

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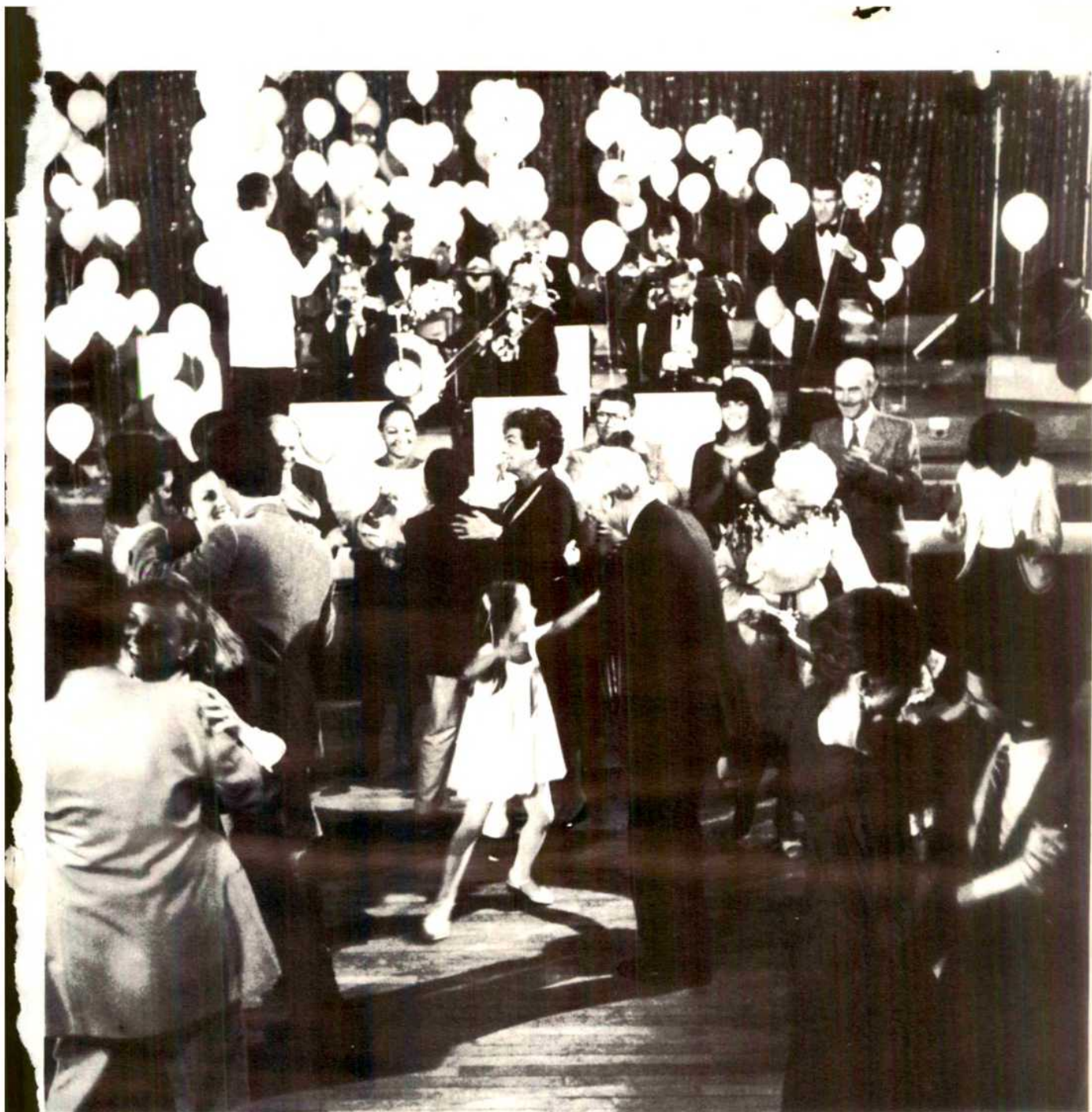
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This position is a temporary, non-tenure track faculty appointment with a major research and teaching commitment. Applicants should send their curriculum vitae and three letters of recommendations to:

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Box 198 UMHC
Department of Laboratory Medicine and Pathology
University of Minnesota Hospital and Clinic
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Minneapolis, MN 55455

Deadline for receipt of applications is March 31, 1987. The University of Minnesota is an equal opportunity educator and employer and specifically invites and encourages applications from women and minorities.



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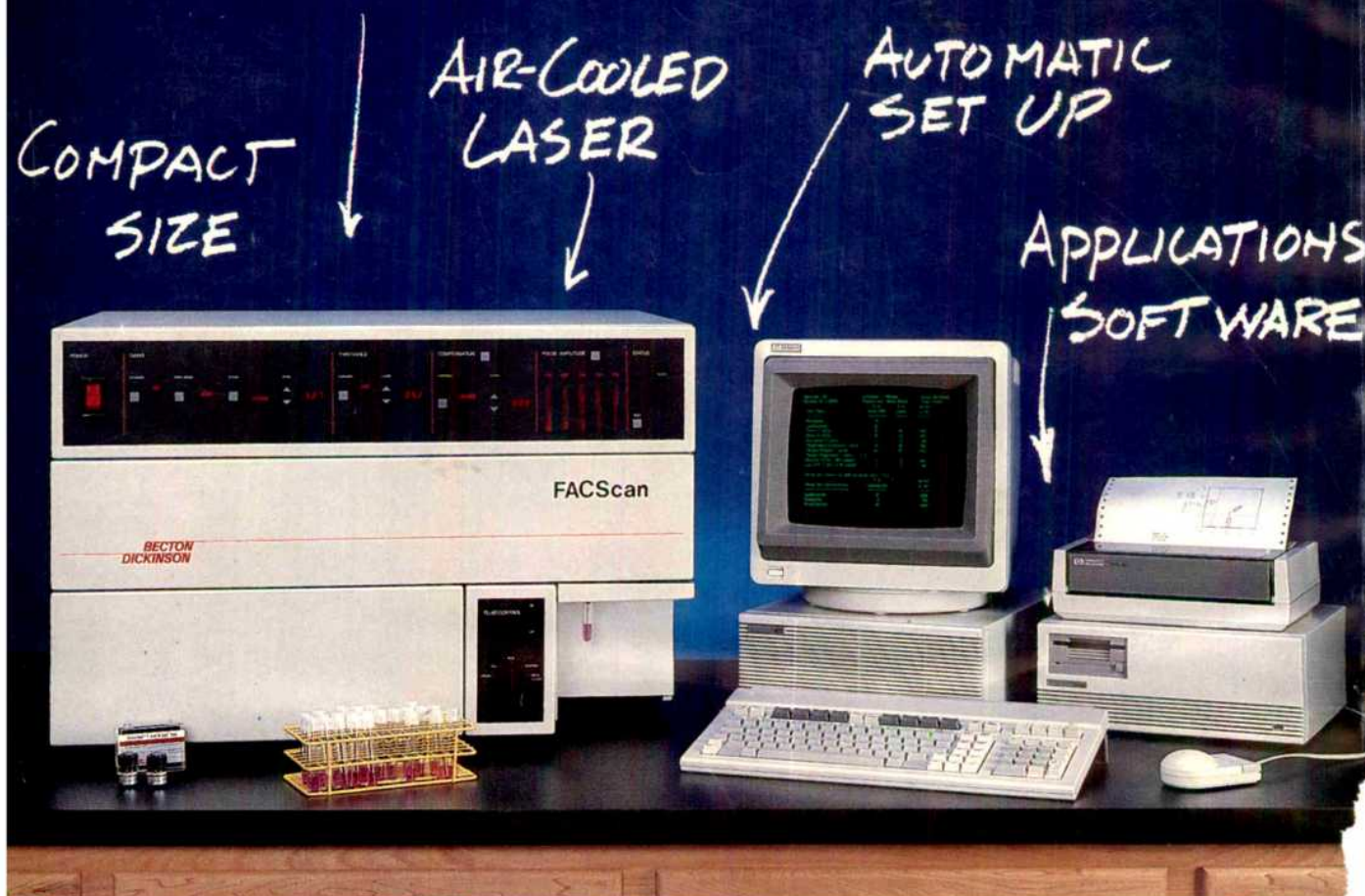
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